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SYMPOSIUM ON EXOERYTHROCYTIC FORMS OF MALARIAL PARASITES

I. INTRODUCTION

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Following the discovery that in certain species of *Plasmodium* the parasites are not restricted to the erythrocytes of the host, it was to be expected that there would be much interest and activity in this field of malariology. The importance of malaria in World War II and the urgency for discovering prophylactic antimalarial agents accelerated the rate at which knowledge of the exoerythrocytic stages was brought forward. Since the function of a symposium is to integrate knowledge in rapidly growing and important fields of research it seemed appropriate to hold a symposium this year on exoerythrocytic forms of malarial parasites. The limitation on time will not permit a detailed account of the history of the development of our knowledge of this subject. Such reviews have already been published and are available to those interested in pursuing the subject (Verney, 1938; Porter & Huff, 1940; Kikuth & Mudrow, 1940; Huff & Coulston, 1944; Huff, 1947). However, for the benefit of those who have not had the opportunity to read these reviews, a few brief summary statements may be in order.

Exoerythrocytic stages are well known only in certain species of *Plasmodium* parasitizing birds and in one species parasitizing lizards. In one of these, *P. elongatum,* almost all cells of the blood and the blood-forming cells of the host are parasitized but the cells of the erythroblastic series are preferred (Huff & Bloom, 1935). In all of the other species of avian malaria which have been adequately studied the cells of the lymphoid-macrophage system are preferred although true endothelium and some other types of cells may be parasitized. In *P. mexicanum* of lizards the potencies of *P. elongatum* and of the species mainly inhabiting the lymphoid-macrophage cells are combined, with the result that any of the cells of the lizard belonging to either of these categories may be parasitized (Thompson & Huff, 1944). In the species *P. gallinaceum, P. relictum, P. cathemerium,* and *P. lophurae* sporozoites and the stages developing from them are known to require a period of development in macrophages and related kinds of cells before they invade the erythrocytes. These stages are collectively called "pre-erythrocytic stages," the first generation following infection by sporozoites being called "cryptozoites" and subsequent generations, "metacryptozoites."

If exoerythrocytic stages exist in the malarial parasites of man and monkeys they are neither as abundant nor as accessible as the comparable stages in avian malaria. Some positive claims have been made for their discovery while other intensive pro-

grams have yielded negative results. The second paper on this symposium will be devoted to a description of the experiments done at Chicago during the war and devoted to attempts to discover such stages in the life cycles of *P. vivax* and *P. cynomolgi*. The indirect evidence favoring the existence of these stages is more convincing. It consists of (1) the existence of stages of inapparent parasitism during which parasitemia in the peripheral blood does not exist followed by frank parasitemia and (2) the evidence derived from the chemotherapy of the disease. The evidence in this latter category will be covered by the third paper in the series.

It is not surprising that the discovery of completely new stages in the life cycle of malarial parasites would provide the means for attacking some old problems in a new way. The facts that exoerythrocytic stages are larger than the erythrocytic stages, that they live in different cells and are capable of being studied by different staining techniques make possible the use of these stages for studying the effects of drugs and of immune reactions of the host upon the parasites. The use of fixation and staining techniques widely used by histologists and pathologists make possible comparisons of what is seen in the parasites with what is known about the tissues of the host. In the fourth paper are presented some of the results of studying the effects of drugs and of immune substances produced by the host upon the exoerythrocytic stages.

The greater ease with which some of the host's cells can be kept alive *in vitro* as compared with erythrocytes permits more readily the use of the former in tissue culture. The results so far obtained by this method are reviewed in the fifth paper of this symposium.

As I have already indicated, we know that in naturally transmitted infections of certain species of avian malarial parasites exoerythrocytic stages play a necessary part in the cycle. The pattern of development of the various stages of the parasite is very constant in such infections. It is, therefore, of importance to know what alterations in these patterns of development follow when the method of transmission or the species of host or age of host are altered. The sixth and final paper of this series deals with the alterations in the pattern of development produced (1) when the infections are transmitted continuously from bird to bird by inoculations of infected blood, (2) when transmissions are made by serial transfer of infected brain from chick to chick, and (3) when the brain tissue from infected chick embryos is transferred serially to other chick embryos.

In introducing this symposium I should like to point out certain limitations which plague the contributors. We do not present it as a completed picture nor as an attempt to answer all of the questions concerning exoerythrocytic stages. We are presenting it as a combination of our own research efforts with a discussion of the pertinent literature in those fields covered by our respective papers. We are specially anxious that our conclusions based on one or more species of malarial parasites be not extrapolated to all such species. We do not know whether many of the findings on avian malaria can be carried over to human or simian malaria. The study of exoerythrocytic stages of malarial parasites is just beginning. We hope that our symposium will stimulate interest in this field. Finally I should like to plead for viewing malariology as a whole and attempting to make it grow in all of its different divisions. From this viewpoint all contributions upon the parasitology of any species of *Plasmodium* should be helpful. Furthermore, the species of the

related genera, *Leucocytozoon* and *Haemoproteus* should not be neglected, for they are almost exclusively exoerythrocytic in their development.

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SYMPOSIUM ON EXOERYTHROCYTIC FORMS OF MALARIAL PARASITES

II. A SEARCH FOR PRE-ERYTHROCYTIC STAGES OF *P. VIVAX* AND OF *P. CYNOMOLGI*

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The indirect evidence that malarial parasites of man and monkeys pass through pre-erythrocytic stages before they begin their erythrocytic development is very persuasive. Several drugs are now known to prevent infection effectively following the inoculation of erythrocytic stages into the uninfected host. However, when the infections are induced by the inoculation of sporozoites there are very few drugs which act prophylactically. Furthermore, there exists a period of inapparent parasitism in sporozoite-induced infections during which the blood of the host is uninfective to other susceptible hosts. Such a period does not exist in blood-induced infections. The prepatent period in such infections is inversely proportional to the size of the inoculum and when the latter is large enough the prepatent period is reduced to a period of minutes. The general acceptance of Schaudinn's (1903) statement that sporozoites enter erythrocytes directly has given away to skepticism as investigator after investigator has failed utterly to confirm his observations. While exoerythrocytic stages of one species of saurian and of several species of avian malaria are well known, the early stages of development in the vertebrate host, i.e., the pre-erythrocytic stages, have been incontestably demonstrated in only four species of avian malaria (*P. gallinaceum, P. cathemerium, P. relictum,* and *P. lophurae*). Direct evidence of the existence of pre-erythrocytic stages of the malarial parasites of mammals has been fragmentary and inconclusive. In 1943 the writers initiated an extensive program of studies of *P. vivax,* using methods which had been very successful in discovering the pre-erythrocytic stages of avian malaria. Later *P. cynomolgi* was included in our program. Although we were unable in this program of studies to demonstrate the stages we were seeking, we believe that a record of our methods and results will be of value to many in attempting to evaluate the con-

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This investigation was carried out under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

Through a cooperative arrangement between the Malaria Research Unit in charge of Dr. Alf S. Alving, Department of Medicine and the authors, while the latter were at the University of Chicago, the former group assumed the responsibility for the clinical care of the patients studied while the latter bred the mosquitoes, supervised their infection and the inoculation of volunteers for both groups. The work was aided by the participation of Army Medical officers assigned to the project by the Surgeon General, U. S. Army, by the hearty cooperation of the officials of the Manteno State Hospital and the Illinois State Penitentiary (Stateville), by Dr. Martin D. Young, U. S. Public Health Service, Columbia, S. C., and by the cooperation of inmate volunteers of the Stateville Penitentiary. We wish particularly to thank Dr. W. R. Kirschbaum, of the Manteno State Hospital, Capt. Ralph Jones, Jr., and the Orlando laboratory of the Bureau of Entomology, U. S. Department of Agriculture. Mrs. Dorothy F. Marchbank, Misses Theodora Bodé, Charlotte Getz, Beryl Liska, Sophie Stevens, Helen Jordan, Tsugiye Shiroishi, Madeline Lindauer and Patricia Hergenrother rendered invaluable assistance to the project.

flicting statements now in the literature concerning these stages and possibly in guiding others to their demonstration.

MATERIALS AND METHODS

Parasites.—One strain of *P. cynomolgi* and three of *P. vivax* were used, the latter being the McCoy, the St. Elizabeth, and the Chesson strains.

Mosquitoes.—*Anopheles quadrimaculatus* was used as host to both species of *Plasmodium*. In the growing of these mosquitoes one room was used for oviposition and two for the growth of the larva. Adult mosquitoes were stored in flight cages (45 × 45 × 45 cm) in the room kept at 23.5° C and at 70% to 90% relative humidity. The high humidity was one of the most important factors in the production of plentiful oviposition. Glucose solution was supplied to the adult mosquitoes in these cages and immobilized guinea pigs were placed daily in the cages to serve as a source of blood meals. Small pans of water served as places for oviposition in each cage. The eggs were collected daily and transferred to white pans (10 × 28 × 58 cm) in rooms maintained at 24° C, but in which no attempt was made to maintain any humidity control. The eggs were floated inside cork rims (5 × 8 cm) and the water was seeded with protozoan cultures. A variety of finely ground animal foods was found to be successful, including rabbit and dog chows and chicken mash. These substances were sprinkled on the surface of the cultures three times daily. An attempt was made to avoid pellicle formation or a too viscous medium. Pupae were picked daily by hand and placed in emergence chambers. The latter consisted of standard crystallizing dishes 7.5 cm in diameter and 5 cm deep and a specially constructed plastic globe 7.5 cm in diameter and with two polar openings 5.5 cm in diameter. The upper opening of the globe was covered with gauze held in place by a metal band. At times an older type of emergence chamber was used. These consisted of lantern globes covered on the upper end with gauze and resting on crystallizing dishes 9 cm in diameter and 5 cm deep. The adults emerged into the globes and were confined in the latter by placing gauze over the lower opening. The globe cages were then placed in pans in the bottom of which there was a layer of moistened cellucotton and stored in constant temperature incubators at 25° C for 4 to 5 days during which time they were fed glucose (about 2%) solution. Twenty-four hours prior to feeding they were kept away from access to liquids. Only the plastic cages were used for applying mosquitoes to patients since the use of glass cages would be attended by danger of breaking the cage and releasing mosquitoes in the hospital wards. The mosquitoes were transported to and from the hospitals in a large balsa wood box which was well enough insulated to require little or no refrigeration in summer or heat in the winter. Cages containing approximately 15 female mosquitoes were applied to the legs of the patient. Engorgement usually was complete in 15 minutes. The unengorged mosquitoes either died in 24 hours if kept away from food or were killed by forceps with specially constructed jaws by reaching through the gauze with the forceps and crushing the mosquitoes. The fed females were stored in incubators (25° C) for 6–7 days and then sampled for stomach infections. If sufficiently well infected, they were further incubated and sampled at 12–14 days for salivary gland infections.

Dissection and Inoculation of Sporozoites.—The infected mosquitoes were dissected in a solution of one part distilled water and one part serum of the subject to

be inoculated (man or monkey). Sterile instruments, slides, and solutions were used during the dissection. Methods for handling, concentration, and inoculation into skin of sporozoites of both *P. vivax* and *P. cynomolgi* were similar to those already described (p. 235, Huff and Coulston, 1944) for *P. gallinaceum*. In the case of isolated veins and lymph nodes of man and of liver, spleen, and bone marrow of monkeys these organs were exposed by operative technics and the infected salivary glands were inoculated superficially into the organs and then the wound was closed.

Biopsy.—The procedures followed were similar to those for *P. gallinaceum* except that standard operative procedures were followed. This tissue was cut with

TABLE 1.—*Biopsy data on human subjects receiving inoculations of sporozoites of* P. vivax

Biopsy No.	Patient designation	Date of inoculation	Strain of *P. vivax*	Size and kind of inoculum		Date of biopsy	Interval (in hours) between inoculation and biopsy	Prepatent period (days)
				**	†			
1	RO*	31–VII–'43	McCoy	6	Sc	1–VIII–'43	96	Neg.
2a	FR	19–VIII–'43	"	20	Sc	23–VIII–'43	96	+
2b	FR	21–VIII–'43	"	18	Sc	23–VIII–'43	48	+
3	Mo	3–XI–'43	"	30	Sc	5–XI–'43	48	+
4	Ce	17–XI–'43	"	30	Sc	18–XI–'43	24	12
5	Da	22–XI–'43	"	30	Sc	24–XI–'43	48	18
6a	Be	20–XII–'43	"	20	Sc	21–XII–'43	23	17
6b	Be	20–XII–'43	"	17	Sc	21–XII–'43	23	
7a	AN	5–II–'44	"	30	Sc	8–II–'44	72	15
7b	AN	5–II–'44	"	30	Sc	8–II–'44	72	
8	Ja	5–II–'44	"	25	Sc	8–II–'44	72	15
9a	Rh	21–IV–'44	St. Elizabeth	35	Sc	25–IV–'44	90	+
9b	Rh	21–IV–'44	" "	30	Sc	25–IV–'44	90	+
10	Kl	25–IV–'44	" "	35	Sc	27–IV–'44	48	+
11a	MK	25–IV–'44	" "	30	Sc	27–IV–'44	48	+
11b	MK	25–IV–'44	" "	30	Sc	27–IV–'44	48	+
12a	Mg	27–IV–'44	" "	30	Sc	28–IV–'44	21	***
12b	Mg	27–IV–'44	" "	30	Sc	28–IV–'44	20	***
13	MI	29–VI–'44	" "	40	Sc	29–IV–'44	2	+
14	Fg	29–VI–'44	" "	30	LN	1–VII–'44	48	+
15	Ei	29–VI–'44	" "	30	LN	1–VII–'44	48	+
17a	Ze	9–III–'45	Chesson	50	Sc	11–III–'45	48	13
17b	Ze	9–III–'45	"	50	LN	12–III–'45	72	..
17c	Ze	10–IV–'45	"	50	LN	13–IV–'45	72	..
18a	Mr	24–III–'45	"	35	Sc	26–III–'45	48	12
18b	Mr	24–III–'45	"	50	LN	30–III–'45	150	
20	Mc	10–VII–'45	"	50	IM	12–VIII–'45	48	Neg.
21	Ru	27–VIII–'45	"	60	LN	31–VII–'45	97	14
22	Ba	8–VIII–'45	"	40	LN	10–VIII–'45	48	13
25a	Mn	22–XI–'45	"	70	Sc	25–XI–'45	72	13
25b	MN	22–XI–'45	"	35	Sc	27–XI–'45	120	
26a	Ga	27–XI–'45	"	20	Isol. vein	29–X–'45	48	11
26b	Ga	27–XI–'45	"	20	SC	29–XI–'45	48	
26c	Ga	27–XI–'45	"	20	Isol. vein	1–XII–'45	96	
27a	Si	29–XI–'45	"	40 bites	BM	10–XII–'45	11 (days)	12
27b	Si	29–XI–'45	"	"	BM	12–XII–'45	13 (days)	
28a	Ir	27–XII–'45	"	16	Isol. vein	29–XII–'45	48	14
28b	Ir	27–XII–'45	"	16	"	31–XII–'45	96	

* Female. All other patients were males.
** Numbers of infected mosquitoes used.
*** Treated with quinacrine during prepatent period.
† Sc = subcutaneous; LN = Lymph node; Isol. vein = Isolated vein; IM = Intramuscular; BM = Bone marrow.

razor blades to blocks measuring no more than 5 mm in its thinnest dimension and fixed in Zenker-formalin for 4 to 6 hours.

Preparation of Stained Slides.—The same as for *P. gallinaceum* (Huff and Coulston, 1944).

Human Subjects.—The first 16 human subjects were general paresis patients in the Manteno State Hospital who were given malaria for therapeusis. The remainder of the subjects were healthy volunteers in the Illinois State Penitentiary at Stateville. All feedings for the purpose of infecting mosquitoes were made on patients in the Manteno State Hospital where the strains of malaria were maintained by blood inoculation.

Monkeys.—Macaca mulatta (rhesus monkeys) were used for the experiments with *P. cynomolgi.* The following species were used in the attempt to find the pre-erythrocytic stages of *P. vivax* in abnormal hosts: *Papio papio* (baboon), *Cercopithecus aethiops pygerythrus, Cercopithecus aethiops sabaeus, Cercocebus fuliginosus,* and *Macaca mulatta.*

MICROSCOPICAL SEARCH FOR PRE-ERYTHROCYTIC STAGES OF *P. vivax* IN HUMAN VOLUNTEERS

We began our experiments on human subjects soon after we had successfully demonstrated the pre-erythrocytic stages of *P. gallinaceum* and continued them concomitantly with further studies on this species as well as on *P. cathemerium, P. relictum,* and *P. lophurae.* The work upon avian malaria indicated that an appreciable proportion of the sporozoites inoculated into the skin developed into cryptozoites locally at the point of inoculation and that after the first generation of growth the pre-erythrocytic stages were found easily in many other organs. This work also indicated that these pre-erythrocytic stages required approximately the same amount of time to undergo a generation as was required for the erythrocytic schizogony to be completed. These findings guided our early attempts to discover pre-erythrocytic stages of *P. vivax* both in the manner and location of the inoculation and in the time for leaving the sporozoites in the skin before biopsying the area. The biopsied area of skin or other organ was carefully sectioned from top to bottom and all sections were saved.

The data relating to the tissues studied are given in Table 1. Twenty-three male and one female patients were inoculated each with inoculums varying from one to three in number and from the salivary glands from 6 to 150 infected mosquitoes. A total of 38 biopsies were made and these were from the following organs:

Skin	24
Lymph nodes	7
Isolated veins	4
Bone marrow	2
Muscle	1

The mean number of mosquitoes used per biopsied area was 30.2 ± 2.5. The intervals of time between the inoculation and the biopsy ranged from 2 hours to 13 days but the majority of these intervals were 24, 48, 72, and 96 hours.

It was not possible to obtain accurate records on the first day that parasitemia appeared after sporozoite inoculation in all of the patients in the Manteno State Hospital. There were two patients who showed no parasitemia and one to whom quinacrine was given during the prepatent period. In the 13 patients showing parasitemia the mean prepatent period was: 14.6 ± 1.2 days. The coefficient of correlation between the numbers of mosquitoes used for inoculums per patient and the length of the prepatent period was -0.516 ± 0.178. (This is based on calculations from data in both Table 1 and Table 2.)

The results of our microscopical examination of these tissues were negative. We examined 24 areas of skin previously inoculated with the sporozoites from 6 to 50 mosquitoes and biopsied at intervals of 2, 20, 24, 48, 72, 96, and 120 hours. Some of these areas had been injected about 8 days previously with 1: 10,000 dilu-

TABLE 2.—*Inoculation and transplantation experiments from patients receiving sporozoites of* P. vivax (*Chesson strain*)

Patient receiving sporozoites (donor)	Number infected mosquitoes	Manner of inoculation	Date of inoculation	Date of subinoculation or transplantation	No. hours between inoculation and removal of blood or skin	Material transferred	Method of transfer	Recipient of biopsied tissues	Parasitemia in recipient	Prepatent period in donor
Ma	50	Subcut.	18–V–'45	21–V–'45	72	Skin	Sc.I.	Ka	Neg.	12 days
Le	25	"	19–V–'45	21–V–'45	48	Skin	Sc.I.	Sc	Neg.	" "
Le	25	"	20–V–'45	21–V–'45	24	Skin	Sc.I.	Pi	Neg.	" "
Bk	40	Mosq. bites	30–IV–'46	2–V–'46	40	Skin	M.P.G.	Dy	Neg.	11 days
Bk	..	" "	" " "	2–V–'46	40	200 ml. blood	I.V.	Wi	Neg.	" "
Bk	40	Mosq. bites	30–IV–'46	3–V–'46	74	Skin	M.P.G.	Lo	Neg.	" "
Bk	..	" "	" " "	3–V–'46	74	200 ml. blood	I.V.	Hy	Neg.	" "
Me	40	Mosq. bites	2–V–'46	2–V–'46	6	Skin	M.P.G.	My	Neg.	9 days
Me	40	Mosq. bites	2–V–'46	3–V–'46	24	Skin	M.P.G.	Pa	Neg.	" "

Sc.I. = Subcutaneous implantation.
M.P.G. = Multiple pinch grafts.
I.V. = Intravenous inoculation.

tion of old tuberculin in order to stimulate macrophage response. In our examination of the sections of skin we were, in a large proportion of cases, able to locate debris from the inoculum, i.e., mosquito scales, tracheae, and salivary ducts from salivary glands. It was in similar areas that we found abundant pre-erythrocytic stages in our studies of material from birds inoculated with the four species of avian *Plasmodium*.

The experiments involving inoculation of sporozoites directly into inguinal lymph nodes were undertaken after a considerable number of portions of skin which had been inoculated with sporozoites had been examined without success. The seven lymph nodes received sporozoites from 30 to 60 mosquitoes and were biopsied

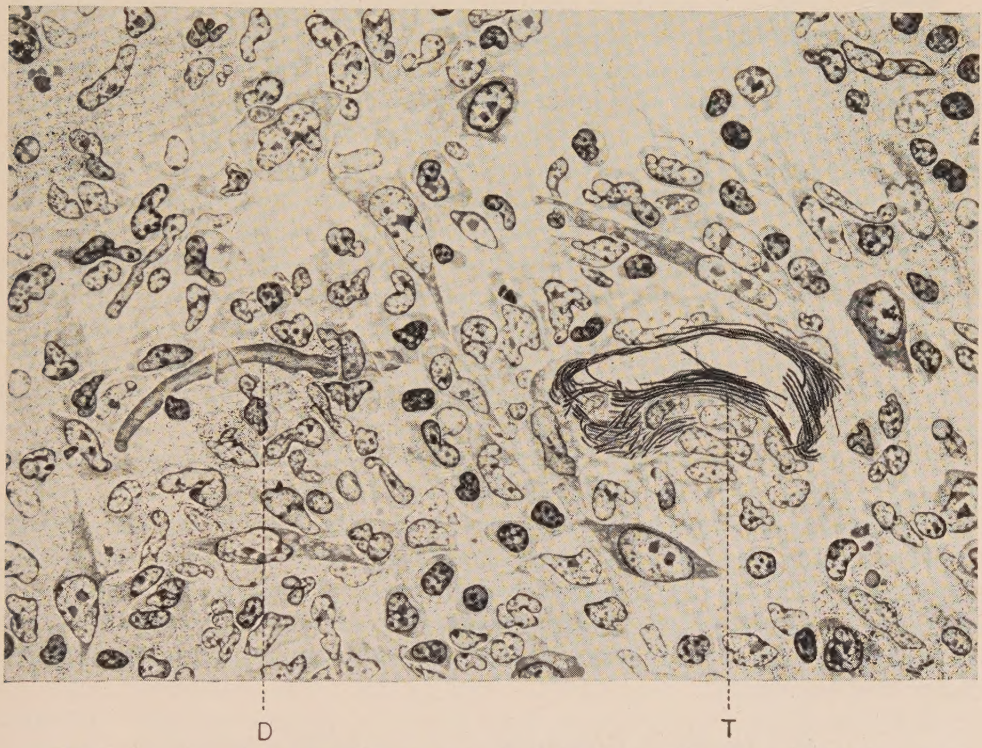

FIG. 1. Section of a human lymph node fixed 97 hours after direct inoculation of the salivary glands from 60 *Anopheles quadrimaculatus* infected with *Plasmodium vivax*. *D*, the remains of the duct from a salivary gland; *T*, oblique section of trachea which was probably attached to the salivary gland when the inoculation was made. Note cells of the lymphoid-macrophage series in the vicinity of the inoculated material. Magnification: ×875. Drawn by Mrs. Esther B. Patterson.

at intervals of 48, 72, 97, and 150 hours. The negative findings from this material were particularly impressive since remnants of mosquito tissues were found surrounded with many types of cells which might be presumed to be hosts to the hypothetical pre-erythrocytic stages of *P. vivax*. Fig. 1 portrays the reaction of a human lymph node 97 hours after inoculation. Mosquito trachea and salivary duct are visible. Macrophages and fibroblasts, as well as more primitive lymphoid cells, are in their immediate vicinity.

The experiments with isolated veins into which sporozoites were injected tested whether or not sporozoites could enter the endothelial cells of the blood vessel.

Again, our findings were negative. The single muscle biopsy was of dubious importance since the patient did not develop malaria after inoculation. The examinations of bone marrow were made following a total of 80 mosquito bites made on the skin. Sternal marrow was obtained by puncture on the 11th and 13th days. Wet-fixed smears were made and small portions of bone marrow were fixed and sectioned. The patient had parasitemia on the 12th day. No exoerythrocytic stages were found in any of these preparations.

SUBINOCULATION EXPERIMENTS EMPLOYING TISSUES INOCULATED WITH *P. vivax* SPOROZOITES

After a considerable number of negative microscopical findings had been made on areas of skin into which sporozoites of *P. vivax* had been inoculated, experiments were devised to test by subinoculation whether parasites were present in the inoculated area. Two patients were given subcutaneous inoculations of sporozoites by the method followed for areas to be biopsied and each of two patients were bitten by 40 infected *Anopheles quadrimaculatus* in each of two areas of skin of the thigh. A concentration of the bites to an area about one-half inch in diameter was effected by covering the skin with adhesive tape in which a hole of that size had been cut. The first three transfers of inoculated skin (from patients *Ma* and *Le*) were subcutaneous implantations. For these implantations the biopsied area was cut in two parts, one of them was minced and the other left intact and both halves were implanted in this form. All other skin transfers were by multiple pinch grafts. In patient *Dy* one-half of the grafts remained viable, in patient *Lo* all remained viable and in patients *My* and *Pa* seven out of nine remained viable.

The results were consistent. The three donors of biopsied material (i.e., those persons receiving sporozoites) became infected in 9 to 12 days. None of the patients receiving donor skin (or blood) became infected. The intervals of time elapsing between entry of sporozoites and biopsy of the bitten area were: 6, 24, 40, 48, 72, and 74 hours. These results together with the negative microscopical findings reported above indicate strongly that sporozoites of *P. vivax* do not remain viable or develop into pre-erythrocytic stages at the site of inoculation in man more than 6 hours after inoculation.

The Attempt to Demonstrate Pre-erythrocytic Stages of P. vivax *in Monkeys and a Baboon**

Although adaptation of *P. vivax* to monkeys has never been successful enough to allow the easy maintenance of the strain through serial blood passage there seemed to be justification for attempting to find the pre-erythrocytic stages in these animals. We (1946) demonstrated that by the inoculation of sporozoites of *P. gallinaceum* and *P. relictum* into species of hosts in which no parasitemia resulted it was possible in certain instances to demonstrate pre-erythrocytic stages microscopically in these animals. Since we were unable to do the kind of experiments on man which would explore the internal organs as the site of possible pre-erythrocytic stages we turned to the use of several species of monkeys. Table 3 gives the data relating to the

* Since this symposium was given the English protozoologists, Shortt and Garnham, have demonstrated pre-erythrocytic stages of *P. cynomolgi* in the liver of monkeys, and Shortt, Garnham, Covell, and Shute have found similar stages of *P. vivax* in human liver. Prolonged researching of our sectioned material has resulted in the discovery of structures presumed to be

material obtained from biopsies of monkeys inoculated with sporozoites of *P. vivax*. Liver, spleen, bone marrow, and skin were the organs tested. Prolonged searching of the sections of these organs did not yield any pre-erythrocytic stages. We cannot conclude from these experiments that possible pre-erythrocytic stages of *P. vivax* do not live in similar organs of man. They only show that any such hypothetical stages do not grow in abnormal hosts in the manner shown for *P. gallinaceum* and *P. relictum*.

TABLE 3.—*Data on biopsies of monkeys inoculated with sporozoites of* P. vivax *(Chesson)*

Species and number	Place of inoculation of sporozoites	No. infected mosquitoes used	Date of inoculation	Date of biopsy	Time between inoculation and biopsy	Parasitemia and pre-erythrocytic stages
Macaca mulatta X28	Skin	35	19–III–'45	21–III–'45	39 hrs.	None
" " X38	Skin	50	23–IV–'45	26–IV–'45	72 hrs.	None
" " A	Bone marrow	50	24–V–'45	28–V–'45	96 hrs.	None
" " X48	Bone marrow	25	5–VI–'45	7–VI–'45	48 hrs.	None
" " X48	Liver	30	5–VI–'45	7–VI–'45	48 hrs.	None
" " X50	Skin	75	12–VI–'45	14–VI–'45	48 hrs.	None
Cercopithecus aethiops sabaeus I	Bone marrow	30	7–VI–'45	8–VI–'45	23 hrs.	None
" " I	Spleen	30	3–VII–'45	5–VII–'45	46 hrs.	None
" " I	Skin	35	2–IX–'45	4–IX–'45	43 hrs.	None
" " I	Skin	35	2–IX–'45	4–IX–'45	45 hrs.	None
Cercopithecus aethiops pygerythrus II	Liver	35	24–VII–'45	26–VII–'45	43 hrs.	None
" " II	Spleen	35	24–VII–'45	26–VII–'45	43 hrs.	None
Cercocebus fuliginosus 526	Liver	35	31–III–'45	4–IV–'45	96 hrs.	None
" 526	Spleen	35	31–III–'45	4–IV–'45	96 hrs.	None
Papio papio	Liver	30	15–IX–'45	17–IX–'45	44 hrs.	None

Attempts to Demonstrate Pre-erythrocytic Stages of P. cynomolgi *in* Macaca mulatta

When our various attempts to find pre-erythrocytic stages of *P. vivax* had failed and the possibility of doing the kind of experiments on man which were necessary to reveal these stages appeared to be remote, we turned our attention to *P. cynomolgi*, which resembles *P. vivax* in many ways and is transmissible by the same mosquito, *Anopheles quadrimaculatus*. Ten biopsies were made on 6 *Macaca mulatta* and the organs represented were: spleen, liver, humeral bone marrow, and skin (see Table 4). The time intervals between inoculation and biopsy were 24, 50, 70, and 72 hours. Four of the monkeys were killed at biopsy; the other two had prepatent periods of 5 and 10 days. Since monkey C–14 received the sporozoites from 50 mosquitoes each in liver and spleen and had a prepatent period of 10 days whereas monkey F–7 received the sporozoites from only 50 mosquitoes in the sternal marrow and had a prepatent period of only 5 days, one might suspect that the bone marrow may be the site in which the pre-erythrocytic stages develop. However, it would

cryptozoites of *P. vivax*. They were found in the liver of *Macaca mulatta* into which 30 sets of infected salivary glands of *Anopheles quadrimaculatus* has been inoculated. The biopsy of the injected area was made 48 hours after inoculation. The parasites are fully grown schizonts and resemble very closely the stages of *P. cynomolgi*, described by the above authors and also by Hawking. Sections kindly supplied by the British workers have made possible a close comparison of their findings with ours. If confirmed, this finding proves (1) that pre-erythrocytic stages of *P. vivax* can develop in *Macaca mulatta* and (2) that the life-cycle is 48 hours in the tissues as in the blood. The former was indicated by our (1946) work on avian malaria. The latter is contrary to the belief of Shortt and Garnham that the cycle requires eight days to be completed.

TABLE 4.—*Data on biopsies of* Macaca mulatta *inoculated with sporozoites of* P. cynomolgi

Monkey No.	Place of inoculation of sporozoites	No. infected mosquitoes used	Date of inoculation	Date of biopsy	Time between inoculation and biopsy	Prepatent period (days)	Pre-erythrocytic stages
C–14	Spleen	50	12–IV–'46	15–IV–'46	72 hrs.	10	None
C–14	Liver	50	12–IV–'46	15–IV–'46	72 hrs.	"	None
C–15	Humeral bone marrow	75	23–IV–'46	25–IV–'46	50 hrs.	K*	None
X–49	Liver (a)	26	6–V–'46	7–V–'46	24 hrs.	K*	None
X–49	Liver (b)	26	6–V–'46	7–V–'46	24 hrs.	"	None
F–7	Humeral bone marrow	50	10–V–'46	13–V–'46	72 hrs.	5	None
274	Spleen	53	21–V–'46	22–V–'46	24 hrs.	K*	None
271	Liver	50	31–V–'46	2–VI–'46	70 hrs.	K*	None
271	Spleen	50	31–V–'46	2–VI–'46	70 hrs.	K*	None
271	Skin	11	31–V–'46	2–VI–'46	70 hrs.	"	None

* Killed at the time that tissues were taken.

be unwise to draw such a conclusion on experiments involving only two animals.

The microscopical findings upon the biopsied tissues were uniformly negative.

DISCUSSION

In evaluating the foregoing experiments, it is necessary to stress the limitations imposed by experiments on man. Since the sporozoite suspensions which were to be injected into human subjects could not be tested for bacterial sterility, it was inadvisable to make inoculations into many of the tissues which needed to be studied. And even if we could have assured ourselves of the bacterial sterility of the inoculums, the surgery necessary to successful injection into many of the organs and to the subsequent biopsy of the injected part were not compatible with safety to the volunteers. Our great hope was that the pre-erythrocytic stages which we were seeking would be found in superficial tissues as they had proved to be in the species of avian parasites already studied. We believe we are justified, on basis of our extensive search, to state that it is very improbable that the pre-erythrocytic stages of *P. vivax* develop in the skin of man. Our series of biopsied lymph nodes was smaller than that of skin and our microscopical findings from them were also negative. If cells of the reticulo-endothelial system are the cells which act as hosts of the hypothetical pre-erythrocytic stages, it would appear that we gave them excellent opportunities to become infected. In many areas like the one shown in Fig. 1, the remains of the inoculated salivary glands of mosquitoes consisting of tracheae and accompanying scales indicated that the inoculum containing sporozoites was surrounded by great numbers of cells of the type known to act as host cells to avian malaria.

Although the technics used were identical with those so successful in revealing the pre-erythrocytic stages in avian malaria the objection might be raised that the pre-erythrocytic stage of *P. vivax* could possibly be so different in staining reactions from those of avian malaria that similar technics might reveal the latter and not the former. We believe that the results of our experiments on subinoculation of tissues which had been inoculated with sporozoites strengthens the view that pre-erythrocytic stages were absent from the areas of human skin into which sporozoites had been inoculated. It should again be mentioned that such subinoculation experiments were previously successful when they involved the skin and other organs of chickens inoculated with sporozoites of *P. gallinaceum.*

The experiments on monkeys were designed to circumvent the limitations per-

taining to experiments on human subjects. We had already demonstrated the development of pre-erythrocytic stages of *P. gallinaceum* in ducks although parasitemia rarely followed inoculation of sporozoites into these animals. It seemed logical to attempt to demonstrate pre-erythrocytic stages of *P. vivax* in monkeys although we knew that parasitemia would not result from the inoculation of sporozoites of this species. By this method we were able to investigate some tissues which could not be included in our study of human tissues, namely, liver and spleen. If these experiments had yielded positive results they would have been very significant. Unfortunately our negative results could either mean that pre-erythrocytic stages of *P. vivax* do not grow in the organs tested or that the species of monkeys used could not act as hosts even to the pre-erythrocytic stages.

The experiments with *P. cynomolgi* in *Macaca mulatta* were not open to the objection raised above, for this species of parasite is capable of producing blood infections in these monkeys through inoculation of the sporozoite stage. The infections in *Anopheles quadrimaculatus* with *P. cynomolgi* were comparable in degree to those with *P. vivax*. In all inoculated animals which were not sacrificed, parasitemia followed the inoculation, demonstrating that viable sporozoites had been used. In fact, the prepatent period in one animal was only 5 days. It would, therefore, seem that the experiments were done under conditions very favorable to the localization and demonstration of pre-erythrocytic stages in those organs studied. The number of animals was small, however, and considering the odds against finding a small area of inoculation or parasites which had dispersed, it is not possible to state with certainty that the organs of the monkeys examined (spleen, liver, bone marrow, and skin) are not the sites of development of the pre-erythrocytic stages. In fact, Garnham (1947) reports success in finding the pre-erythrocytic stages of *P. kochi* in the liver of *Cercopithecus* monkeys. The bodies reported by him are large enough that they would not easily be missed. We are, therefore, inclined to believe that the liver is not the site of development of the pre-erythrocytic stages of *P. cynomolgi* or that such stages are smaller than the corresponding forms reported by Garham for *P. kochi*.

One of the surprising results of our work with human and simian subjects was the almost total lack of sepsis at the site of inoculation of sporozoites. As already noted, the need for the prompt inoculation of the sporozoites following the dissection of the infected mosquitoes precluded the possibility of making tests for their bacterial sterility. It is very certain that bacteria and possibly other microorganisms were present in the inoculums yet their presence in the inoculation site was not revealed either by gross lesions or by histologic examination. This was also true of our earlier work with inoculation into chickens of similar material from *Aedes aegypti* infected with *P. gallinaceum*. We were not surprised by the lack of sepsis in these animals because they are notoriously resistant to most environmental microorganisms. We have been led to suspect that some bactericidal substance in the salivary glands of the mosquitoes either inhibited the growth of the contaminating bacteria directly or in some way stimulated the host's tissues to overcome the microorganisms. Duncan (1926) demonstrated the presence in the alimentary tracts of certain blood-sucking arthropods, including mosquitoes, of a bactericidal principle which was very effective in killing just the type of microorganism likely to be introduced by the inoculums of sporozoites which we used. The action of allantoin and urea in pro-

moting healing processes was demonstrated by Simmons (1935) and Robinson (1935, 1937) through a study of the effects of excretions of maggots on wounds.

In spite of our negative findings in these experiments we wish to emphasize that we believe it probable that pre-erythrocytic stages of human and simian malarial parasites exist. The question as to whether others have successfully demonstrated their existence is one which we cannot discuss in detail here. One of us has reviewed the literature on this question elsewhere (Huff, 1947, 1948) and further consideration is given to the indirect evidence in the paper following this one by Coatney and Cooper.

SUMMARY

The microscopical search for pre-erythrocytic stages of *P. vivax* in biopsied areas of skin, lymph nodes, veins, bone marrow, and muscle of human volunteers into which had been inoculated the salivary glands of 6 to 70 infected mosquitoes met with no success.

Transplantation of areas of skin previously inoculated with sporozoites of *P. vivax* to susceptible volunteers failed to produce infection in the recipients of the grafts. The graft donors uniformly became infected.

Microscopical search for pre-erythrocytic stages of *P. vivax* in organs of five species of monkeys failed to reveal evidence of their presence.

No evidence was found of pre-erythrocytic stages of *P. cynomolgi* in organs of *Macaca mulatta* inoculated with sporozoites from 11 to 75 infected *Anopheles quadrimaculatus*.

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SYMPOSIUM ON EXOERYTHROCYTIC FORMS OF MALARIAL PARASITES

III. THE CHEMOTHERAPY OF MALARIA IN RELATION TO OUR KNOWLEDGE OF EXOERYTHROCYTIC FORMS

G. Robert Coatney and W. Clark Cooper[1]

As early as 1893, Golgi suggested that parasites of human malaria may develop in cells where they are not affected by antimalarial drugs, and the idea of a hidden or exoerythrocytic stage has reappeared on numerous occasions in the literature. By 1931, James had access to sufficient data to be able to predict with some assurance that the malaria parasite has a period of development in fixed tissue cells and this concept has been widely accepted to explain such phenomena as the prepatent period, relapse and the inability of certain drugs to cure established avian and *vivax* infections.

Although exoerythrocytic stages had been seen and figured before James' prediction (i.e., Anschütz, 1909, 1910), their significance was not realized until Huff and Bloom (1935) showed these forms to be an integral part of the life cycle of *Plasmodium elongatum*. In 1937, James and Tate (1937; 1937*a*) discovered E-E forms in the brain capillaries of chickens infected with *P. gallinaceum*, but it remained for Huff and his co-workers (Huff and Coulston, 1944; Coulston, Cantrell and Huff, 1945; Huff and Coulston, 1946; Coulston and Huff, 1947; Huff et al, 1947; Huff, 1947; Coulston and Huff, 1948), in a series of brilliantly executed studies, to work out the complete exoerythrocytic cycle in this species and, also, in *P. relictum, P. lophurae* and *P. cathemerium* and thereby establish this phase in the life cycle of the avian malarias.

Exoerythrocytic parasites have been reported from both simian and human malaria, but, as pointed out by Huff (1947), considerable doubt exists as to whether the structures were actually E-E forms.[2] Evidence from chemotherapeutic and subinoculation studies strongly favors their existence and, in fact, it is difficult to present a working hypothesis in agreement with the known facts without including these non-circulating parasites.

Evidence for the activity or inactivity of a given drug against exoerythrocytic parasites can come from several types of experiments:

(1) In species and hosts where the existence of E-E stages has been proven, i.e., certain of the avian malarias: (a) morphological changes observed at biopsy, autopsy or in tissue culture; (b) permanent prevention of erythrocytic infection by the action of a drug against any pre-erythrocytic stage, i.e., *prophylaxis;* (c) eradication of an established infection, i.e., *cure.*

(2) In species and hosts where the existence of E-E stages has not been proven, but where they have been inferred from chemotherapeutic and subinoculation studies:

[1] From the Subdivision on Malaria, Division of Tropical Diseases, National Institutes of Health, Bethesda, Maryland.

[2] After this paper was submitted for publication, Shortt and Garnham (1948) and Shortt, Garnham, and Malamos (1948) conclusively demonstrated exoerythrocytic stages in *P. cynomolgi*. In a subsequent paper Shortt, Garnham, Covell, and Shute (1948) also proved the existence of these forms in *P. vivax*.

TABLE 1.—*Compounds, discussed in this paper, for which there is direct or indirect evidence of activity against exoerythrocytic forms of malaria*

Number*	Chemical name	Empirical formula	Other designations
	1. Quinoline derivatives		
SN 971	8-(4-diethylamino-1-methylbutylamino)-6-methoxyquinoline	$C_{19}H_{29}N_3O$	Pamaquine Plasmochin
SN 1452	8-(3-aminopropylamino)-6-methoxyquinoline	$C_{13}H_{17}N_3O$	.....
SN 9972	8-(4-isopropylamino-1-methylbutylamino-5,6-dimethoxyquinoline	$C_{19}H_{29}N_3O_2$	.....
SN 11,191	8-(6-diethylaminohexylamino)-6-methoxyquinoline	$C_{20}H_{31}N_3O$	.....
SN 13,274	8-(4-isopropylamino-1-methylbutylamino-6-methoxyquinoline	$C_{18}H_{27}N_3O$	Isopentaquine
SN 13,276	8-(5-isopropylaminoamylamino)-6-methoxyquinoline	$C_{18}H_{27}N_3O$	Pentaquine
SN 13,429	6-methoxy-8-(1-methyl-4-propylaminobutylamino) quinoline	$C_{18}H_{27}N_3O$	.....
DR 15,302	8-(5-isopropylaminoamylamino)-6-methoxylepidine	$C_{19}H_{29}N_3O$	.....
	2. Sulfonamides: sulfanilamides		
SN 103	N^1-2-thiazolylsulfanilamide	$C_9H_9N_3O_2S_2$	Sulfathiazole
SN 104	N^1-2-pyridylsulfanilamide	$C_{11}H_{11}N_3O_2S$	Sulfapyridine
SN 112	N^1-2-pyrimidylsulfanilamide	$C_{10}H_{10}N_4O_2S$	Sulfadiazine
SN 113	N^1-(4-methyl-2-pyrimidyl) sulfanilamide	$C_{11}H_{12}N_4O_2S$	Sulfamerazine
SN 194	*p*-aminobenzenesulfonamide	$C_6H_8N_2O_2S$	Sulfanilamide
SN 201	Sulfanilylguanidine	$C_7H_{10}N_4O_2S$	Sulfaguanidine
SN 213	N^1-2-pyrazinylsulfanilamide	$C_{10}H_{10}N_4O_2S$	Sulfapyrazine
SN 232	N^1-(4,6-dimethyl-2-pyrimidyl) sulfanilamide	$C_{12}H_{14}N_4O_2S$	Sulfamethazine Sulfamezathine
SN 236	N^4-benzylsulfanilamide	$C_{13}H_{14}N_2O_2S$	Proseptasine
SN 1493	N^1-3-pyridylsulfanilamide	$C_{11}H_{11}N_3O_2S$	.....
SN 2018	N^1-(6-chloro-2-pyrazinyl) sulfanilamide	$C_{10}H_9ClN_4O_2S$	.....
SN 3078	N^1-2-quinoxalylsulfanilamide	$C_{14}H_{12}N_4O_2S$	Sulfaquinoxaline
SN 3179	N^1-(5-chloro-2-pyrazinyl) sulfanilamide	$C_{10}H_9ClN_4O_2S$	.....
SN 3517	N^1-(5-bromo-4,6-dimethyl-2-pyrimidyl) sulfanilamide	$C_{12}H_{13}BrN_4O_2S$	.....
SN 4800	N^1-(5-bromo-4-methyl-2-thiazolyl) sulfanilamide	$C_{10}H_{10}BrN_3O_2S_2$	.....
SN 4801	N^1-(3-methyl-2-pyrazinyl) sulfanilamide	$C_{11}H_{12}N_4O_2S$	.....
SN 5123	*p*-(2-pyrimidylsulfamyl) acetanilide	$C_{12}H_{12}N_4O_3S$	.....
SN 5127	N^1-(5-methyl-1,3,4-thiadiazol-2-yl) sulfanilamide	$C_9H_{10}N_4O_2S_2$	.....
SN 5240	N^1-(4,5,6-trimethyl-2-pyrimidyl) sulfanilamide	$C_{13}H_{16}N_4O_2S$	.....
SN 5291	N^1-(6-bromo-3-pyridyl) sulfanilamide	$C_{11}H_{10}BrN_3O_2S$	.....
SN 5702	N^1-(5-bromo-4-methyl-2-pyrimidyl) sulfanilamide	$C_{11}H_{11}BrN_4O_2S$	.....
SN 7512	N^1-(4-amino-2-pyrimidyl) sulfanilamide	$C_{10}H_{11}N_5O_2S$	.....
SN 8605	N^1-(5-bromo-2-pyrimidyl) sulfanilamide	$C_{10}H_9BrN_4O_2S$	.....
SN 8607	N^1-(5-chloro-2-pyrimidyl) sulfanilamide	$C_{10}H_9ClN_4O_2S$	.....
SN 9657	N^1-(5-chloro-4-methyl-2-thiazolyl) sulfanilamide	$C_{10}H_{10}ClN_3O_2S_2$	.....
SN 9883	N^1-(4-amino-5-bromo-2-pyrimidyl) sulfanilamide	$C_{10}H_{10}BrN_5O_2S$	.....
SN 11,039	4-amino-N-2-pyrimidyl-*o*-toluenesulfonamide	$C_{11}H_{12}N_4O_2S$	.....
SN 11,048	N^1-(5-bromo-2-thiazolyl) sulfanilamide	$C_9H_8BrN_3O_2S_2$	.....
SN 11,051	N^1-(5-chloro-2-thiazolyl) sulfanilamide	$C_9H_8ClN_3O_2S_2$	.....
SN 12,181	3′-sulfamylsulfanilanilide	$C_{12}H_{13}N_3O_4S_2$	.....
SN 12,200	N^1-(5-chloro-2-pyridyl) sulfanilamide	$C_{11}H_{10}ClN_3O_2S$	.....
SN 12,660	N^1-methyl-N^1-2-pyrimidylsulfanilamide	$C_{11}H_{12}N_4O_2S$	.....
DR 15,588	N^1-2-benzoxazolylsulfanilamide	$C_{13}H_{11}N_3O_3S$	.....
DR 15,592	N^1-(5-iodo-2-pyrimidyl) sulfanilamide	$C_{10}H_9IN_4O_2S$	.....
	2. Sulfonamides: metanilamides		
SN 132	6-sulfanilylmetanilamide	$C_{12}H_{13}N_3O_4S_2$	.....
SN 11,435	N^1-2-pyrimidylmetanilamide	$C_{10}H_{10}N_4O_2S$	.....
SN 11,436	N^1-2-pyrazinylmetanilamide	$C_{10}H_{10}N_4O_2S$	.....
SN 11,437	N^1-(5-chloro-2-pyrimidyl) metanilamide	$C_{10}H_9ClN_4O_2S$	Metachloridine
SN 12,192	5-amino-N-(5-bromo-2-pyrimidyl)-*o*-toluenesulfonamide	$C_{11}H_{11}BrN_4O_2S$	.....
SN 12,203	N^1-(5-bromo-2-pyridyl) metanilamide	$C_{11}H_{10}BrN_3O_2S$	.....
SN 12,648	*m*-(5-chloro-2-pyrimidylsufamyl) acetanilide	$C_{12}H_{11}ClN_4O_3S$	.....
SN 12,652	N^1-3-pyridylmetanilamide	$C_{11}H_{11}N_3O_2S$	.....
SN 12,653	N^1-(5-bromo-2-pyrimidyl) metanilamide	$C_{10}H_9BrN_4O_2S$	.....
SN 14,633	N^1-(5-chloro-2-pyrazinyl) metanilamide	$C_{10}H_9ClN_4O_2S$	.....
DR 15,583	N^1-(5-iodo-2-pyrimidyl) metanilamide	$C_{10}H_9IN_4O_2S$	.....
DR 15,585	2-[(dimetanilyl)amino]-5-chloropyrimidine	$C_{16}H_{14}ClN_5O_4$	.....
DR 15,586	3′-(5-chloro-2-pyrimidylsulfamyl) benzanilide	$C_{17}H_{13}ClN_4O_3S$	.....
DR 15,589	N^1-2-benzoxazolylmetanilamide	$C_{13}H_{11}N_3O_3S$	.....
DR 15,590	N^1-(5-bromo-4-methyl-2-pyrimidyl) metanilamide	$C_{11}H_{11}BrN_4O_2S$	.....
DR 15,591	*m*-[(5-chloro-2-pyrimidyl) sulfamyl] phenylurea	$C_{11}H_{10}ClN_5O_3S$	.....
DR 15,593	2-[(bis-N^3,N^3-dimethylmetanilyl) amino]-5-chloropyrimidine	$C_{20}H_{22}ClN_5O_4S_2$	.....
	2. Sulfonamides: miscellaneous		
DR 15,587	N-(5-chloro-2-pyrimidyl)-*m*-nitrobenzenesulfonamide	$C_{10}H_7ClN_4O_4S$	.....

TABLE 1.—(*Continued*)

Number	Chemical name	Empirical formula	Other designations
	3. Naphthoquinones		
SN 3529	2-hydroxy-3-isoamyl-1,4-naphthoquinone	$C_{15}H_{16}O_3$	Hydrolapachol
SN 5090	2-(3-cyclohexypropyl)-3-hydroxy-1,4-naphthoquinone	$C_{19}H_{22}O_3$	.....
SN 5949	2-hydroxy-3-(2-methyloctyl)-1,4-naphthoquinone	$C_{19}H_{24}O_3$	.....
SN 8438	2-(4-cyclohexylbutyl)-3-hydroxy-1,4-naphthoquinone	$C_{20}H_{24}O_3$	.....
SN 8557	2-[3-(decahydro-2-naphthyl)propyl]-3-hydroxy-1,4-naphthoquinone, mixture of stereoisomers	$C_{23}H_{28}O_3$	.....
	4. Biguanides		
SN 12,837	1-(*p*-chlorophenyl)-5-isopropylbiguanide	$C_{11}H_{16}ClN_5$	M.4888 Paludrine Chlorguanide
DR 15,594	1-(*p*-chlorophenyl)-5-methyl-5-isopropyl-biguanide	$C_{12}H_{18}ClN_5$	M.4430
	5. Acridone derivatives		
SN 13,421†	3-heptyl-7-methoxy-2-methyl-4-quinolinol	$C_{18}H_{25}NO_2$	Endochin
DR 15,575	2-methoxy-5,6,7,8-tetrahydro-9(10)-acridone	$C_{14}H_{15}NO_2$	.....
DR 15,595	3-methoxy-7-methyl-5,6,7,8-tetrahydro-9(10)-acridone	$C_{15}H_{18}NO_2$	.....
DR 15,597	7-ethyl-3-methoxy-5,6,7,8-tetrahydro-9(10)-acridone	$C_{16}H_{19}NO_2$	.....
DR 15,598	3-methoxy-5-methyl-5,6,7,8-tetrahydro-9(10)-acridone	$C_{15}H_{17}NO_2$	.....
	6. A small miscellaneous group		
SN 14,658	2,4-diamino-6-heptyl-*s*-triazine	$C_{10}H_{19}N_5$	.....
SN 14,785	2,4-diamino-6-hexyl-*s*-triazine	$C_9H_{17}N_5$	.....
DR 15,584	4-amino-5-bromomethyl-2-methylpyrimidine	$C_6H_8BrN_3$	.....

* "SN" denotes the Survey Number assigned by the Survey of Antimalarial Drugs (see Wiselogle, 1946). "DR" denotes the Drug Repository number assigned by the Drug Repository in the Subdivision on Malaria, Division of Tropical Diseases, National Institute of Health, Bethesda, Maryland. SN 15,297 was the last number assigned by the Survey Office; Drug Repository numbers begin with DR 15,298 and follow in sequence.

† SN 13,421 (endochin) was the parent compound from which the tetrahydroacridones were modeled.

(a) permanent prevention of erythrocytic infection by the action of a drug during the early stages of a sporozoite-induced infection, i.e., *causal prophylaxis;* (b) eradication of an established infection, i.e., *cure.*

Relatively few investigators have actually described morphological alterations of E-E forms resulting from drug action. In studies of *P. gallinaceum,* Coggeshall, Porter and Laird (1944) and Paraense (1946) carried out serial brain biopsies, Tonkin (1946) and Hawking (1947) used stained tissue culture smears and Coulston and Huff (1948) studied fixed tissue preparations of pre-erythrocytic forms.

It is plainly evident from the above that a discussion of the chemotherapy of E-E forms can hardly be limited to the demonstrable visible effects of the drugs. On the other hand, a large amount of data is available from prophylactic and curative studies of avian and human malarias, and these, when properly interpreted, provide strong presumptive evidence of action against exoerythrocytic parasites.

Many thousands of potential antimalarial compounds have been tested against various malarias, but only a relatively few have shown prophylactic or curative abilities and these will be discussed under six general categories, namely: (1) quinoline derivatives; (2) sulfonamides; (3) naphthoquinones; (4) biguanides; (5) acridone derivatives; and (6) a small miscellaneous group (Table 1).

Before proceeding to the discussion of these drugs, certain definitions should be restated. *Prophylaxis* is defined as permanent prevention of erythrocytic infection

by action of a drug upon the sporozoite or any succeeding pre-erythrocytic stage (i.e., cryptozoites and/or metacryptozoites in avian malarias and the corresponding hypothetical early stages in mammalian malarias). *Partial prophylaxis* indicates an incomplete but qualitatively similar type of action, usually manifested by delay in the appearance of erythrocytic parasites. This type of delay is sometimes difficult to differentiate from delay due to the persistence of a slowly eliminated drug acting against erythrocytic parasites. *Cure* connotes complete eradication of the infection by a drug given therapeutically after the infection has become established. In general, this implies activity against late E-E forms (phanerozoites or similar stages), erythrocytic parasites or both.

We will not attempt to give a critical evaluation of the potentialities of each compound in the chemical groups to be discussed but, rather, will use the pertinent data to present the general principles of drug activity.

AVIAN MALARIA

The bulk of the data on chemotherapeutic action in avian malarias studied under conditions favorable to the demonstration of action against E-E stages has been derived from studies against four species of parasite in four different hosts: *P. gallinaceum* in the chick; *P. cathemerium* in the canary; *P. relictum* in the canary; and *P. lophurae* in the chick, canary, duck and turkey.

As Doctor Huff has outlined in his introductory paper (1948), these infections when initiated by sporozoites are characterized by at least two generations of exoerythrocytic development before red-cell stages appear; then, there is a concurrent development of erythrocytic parasites and E-E forms with some of the erythrocytic merozoites developing in exoerythrocytic sites and *vice versa*. An infection induced by erythrocytic forms may develop exoerythrocytic parasites and this infection will be as resistant to cure by antimalarial drugs as one induced by sporozoites. Thus, while the blood-induced avian infections have demonstrable phanerozoites, there is no evidence that the early E-E stages characteristic of the sporozoite-induced infections (i.e., cryptozoites and metacryptozoites) ever occur in the blood-induced infections.

1. *Quinoline derivatives.* Quinine and chloroquine, and the same is true for the acridine derivative quinacrine (atabrine), although possessing marked suppressive activity against the erythrocytic parasites of avian malaria, are not prophylactic nor will they cure an established infection. Furthermore, if the administration of these drugs, even at the limit of tolerance, is started prior to infection by sporozoites or by the inoculation of infected blood and continued indefinitely, patent parasitemia will be prevented but the birds will die as the result of an overwhelming exoerythrocytic infection.

Pamaquine (plasmochin), the best-known representative of the 8-aminoquinolines, was the earliest widely exploited synthetic antimalarial. In view of the fact that representatives of this group show definite causal prophylactic and curative activity in human malaria, it is somewhat surprising that we find such poor evidence for their activity against exoerythrocytic parasites in the avian malarias. In fact, although a great many of these compounds have been studied in birds, not a single one has been found capable of preventing *gallinaceum* malaria in the chick, even at maximum tolerated dosage. Slight delay of infection is the most that has been

achieved. Partial prophylactic action was achieved by Gingrich (1946) with 30 of these compounds against *P. cathemerium* in the canary, and similar results were obtained by Coggeshall and Porter (1946) in tests with 7 of these substances against *P. lophurae* in the turkey.

2. *Sulfonamides.* Prophylactic activity against *gallinaceum* malaria has been demonstrated for over 30 derivatives of sulfanilamide since it was established early in 1942 (Coatney and Cooper, 1944; Coggeshall, Porter and Laird, 1944) that sulfadiazine would prevent this infection. Details and results of the tests can be found in the foregoing reports or in those of Americano Freire and Paraense (1944), Brackett, Waletzky and Baker (1945), Curd, Davey and Rose (1945), Brackett (1946), Coatney and Sebrell (1946), Coggeshall and Porter (1946), Geiling and Taliaferro (1946), Americano Freire (1946), Brackett and Waletzky (1946; 1946a), Davey (1946) and Coatney and Cooper (unpublished).

None of these drugs has produced complete prophylaxis in the other avian malarias; some did produce delay, however, against *P. lophurae* in the chick and the turkey (Coggeshall and Porter, 1946) and against *P. cathemerium* in the canary (Geiling and Taliaferro, 1946; Gingrich, 1946; Hughes and Brackett, 1946).

The greatest relative activity has been shown by the pyrimidine and pyrazine derivatives (e.g., sulfadiazine and sulfapyrazine) of sulfanilamide. Sulfadiazine, because of its early recognition as a prophylactic agent and because of its wide range of effectiveness, was made the primary standard of reference in assessing prophylactic activity of other compounds against *gallinaceum* malaria. It has also been the most widely studied compound of the group in human malaria.

The meta isomers of the sulfanilamides were first tested by Brackett and his co-workers. They have shown that 17 of these compounds (Table 1) are prophylactic against *P. gallinaceum* in the chick and one of them, SN 11,437 (metachloridine), also against *P. cathemerium* in the canary (Brackett, 1946; Brackett and Waletzky, 1946; Hughes and Brackett, 1946; Brackett and Waletzky, 1946a; Brackett, personal communication). Three of the compounds, SN 132, SN 11,435 and SN 11,437, have also been studied by other investigators. SN 132 was found to produce only delay of patent infection in *P. gallinaceum* by Coatney and Sebrell (1946) and by Maier (1946) although Coggeshall and Porter (1946) were able to obtain complete prophylaxis. Against *P. cathemerium* in the canary, Gingrich (1946) reported only slight delay; and Coggeshall and Porter (1946) were unable to obtain any evidence of prophylactic activity against *P. lophurae* in the turkey. SN 11,435 was found by Gingrich (1946) to produce an appreciable delay against *P. cathemerium* in the canary (cf., Hughes and Brackett, 1946), but by Coggeshall and Porter (1946) to produce complete prophylaxis against *P. lophurae* in the turkey. SN 11,437 showed the greatest range of activity, giving complete prophylaxis against *P. cathemerium* in the canary (Gingrich, 1946; Gingrich, Schoch and Taylor, 1946), *P. lophurae* in the turkey (Coggeshall and Porter, 1946) and *P. gallinaceum* in the chick (Coatney and Sebrell, 1946).

Recently, Zuckerman (1946), by exposing exoerythrocytic parasites of *P. gallinaceum* to the action of sulfadiazine *in vitro* and then implanting them on the chorioallantoic membrane of chick embryos, was able to show a marked suppressive effect, as judged by the subsequent infection in these embryos.

Another sulfonamide, DR 15,587, a nitro analogue of metachloridine, has also been found to be prophylactic against *P. gallinaceum* by Brackett (personal communication).

Cures of *P. gallinaceum* infections by sulfadiazine, sulfapyridine and sulfanilamide have been reported by Americano Freire and Paraense (1944) but other investigators, including ourselves, have been unable to obtain similar results even when the drugs were given at the limit of tolerance for long periods.

3. *Naphthoquinones.* Several derivatives of 1,4-naphthoquinone have shown prophylactic activity. Coatney and Sebrell (1946) found that SN 5090, SN 5949, SN 8438 and SN 8557 exhibited complete prophylaxis against *gallinaceum* malaria in the chick. The first, second and last of these compounds produced delay of infections caused by *P. cathemerium* in the canary (Gingrich, 1946) and *P. lophurae* in the turkey (Coggeshall and Porter, 1946).

One of the aforementioned compounds, SN 8557, has been reported as curative against 86 percent of the blood-induced 3C strain treated during latency, and 84 percent of the sporozoite-induced 3H2-1 strain, also treated during latency, of *P. cathemerium* in the canary by Gingrich and his co-workers (Gingrich, 1946; Gingrich et al, 1947). This is pointed out because these are the first authentic cures of established avian infections when exoerythrocytic stages are known to occur.

4. *Biguanides.* Curd, Davey and Rose (1945) have described in considerable detail the chain of investigations which led to their discovery of prophylactic activity in two biguanide derivatives. One of these compounds, M.4430 (DR 15,594), was reported as affording complete protection against *P. gallinaceum* in the chick while the second, M.4888 (SN 12,837), now known as paludrine or chlorguanide, not only was completely prophylactic against this infection but also against *P. cathemerium* and *P. relictum* in the canary and possibly *P. lophurae* in the chick. Davey, in 1946, somewhat modified the previous report, describing complete prophylaxis against *P. gallinaceum* and *P. lophurae* in the chick but only low-grade activity against *P. cathemerium* and *P. relictum* in the canary. Gingrich (1946) reported no activity was obtained against *P. cathemerium* in the canary. In our own laboratory (unpublished data) paludrine has been demonstrated to be a true causal prophylactic against *P. gallinaceum,* but only at toxic levels.

The above results with paludrine against *P. gallinaceum* have been confirmed in tissue cultures by Hawking (1947). It is of considerable interest that paludrine *per se* did not exhibit this activity, but, rather, that the active principle was found in blood drawn from an animal (a fowl) following administration of paludrine, suggesting that the action against exoerythrocytic forms is the result of a metabolic product of paludrine formed by the animal host.

Attempts to cure avian malarias with paludrine have been uniformly unsuccessful.

5. *Acridone derivatives.* When reports on German research on antimalarials during the recent war became available (Combined Intelligence Objectives Sub-Committee, 1945, 1945a, 1945b; Fitch, 1945), it was found that causal prophylactic action against *P. relictum* and *P. cathemerium* in the canary had been obtained by Kikuth with a compound called endochin (3-heptyl-7-methoxy-2-methyl-4-quinolinol) and that Kikuth was very hopeful that a curative antimalarial would be found among related compounds. Endochin (SN 13,421) was eventually tested in the United States, and in our laboratory we found it to be prophylactic against *P. galli-*

naceum (Coatney and Sebrell, 1946), but Gingrich (1946), working with *P. cathemerium* in the canary, and Coggeshall and Porter (1946), working with *P. lophurae* in the turkey, obtained only delay.

When Kikuth's results with endochin came to their attention, Stephen, Tonkin and Walker (1945) hastened to report results which they had obtained with a closely related group of compounds, the tetrahydroacridones. This preliminary report was followed by a more definitive paper in 1947, in which prophylactic activity was described for four tetrahydroacridones against *P. gallinaceum* in the chick, one of these (DR 15,575) being twice as active as either sulfadiazine or endochin.

6. *A small miscellaneous group.* Other compounds have been reported, with supporting evidence, as having activity in bird malaria consistent with effects on exoerythrocytic forms. DR 15,584 (4-amino-5-bromomethyl-2-methylpyrimidine) has been found by Brackett (personal communication) to be prophylactic against *P. gallinaceum* in the chick. Two *s*-triazines (SN 14,658 and SN 14,785), i.e., compounds with three nitrogens arranged symmetrically in the basic ring, have also been reported as prophylactic against *P. gallinaceum* (Brackett and Waletzky, 1946a; Brackett, personal communication). These leads have yet to be exploited.

SIMIAN MALARIA

Very little can yet be adduced from studies in monkey malaria relevant to our knowledge of chemotherapeutic effects on exoerythrocytic stages. The two most widely used parasites for trials of drugs have been *P. knowlesi* and *P. cynomolgi.* The former has so far only been transferred by blood inoculation and there is not even circumstantial evidence that this infection may harbor exoerythrocytic forms. It is interesting in this connection, however, to recall the demonstration by Coggeshall (1938, 1938a) that sulfanilamide would cure *knowlesi* malaria whereas quinacrine or quinine only produce chronicity. It is impossible with our present knowledge, however, to relate this to exoerythrocytic stages.

Anopheline mosquitoes will readily transmit *P. cynomolgi* to the monkey and the résultant infection bears many resemblances to that of *P. vivax* in man. As a consequence, this parasite bears promise as a screening aid prior to the use of potential antimalarial drugs in man. Schmidt (personal communication) is now carrying out the preliminary studies necessary for such an approach. He has tested paludrine, chloroquine, quinacrine and quinine at the limit of tolerance and found none of these drugs to be prophylactic or curative when given alone. Prophylactic trials with pamaquine and pentaquine are not yet complete, but in curative tests it appears that either of these two compounds will eradicate established infections when given alone or in combination with quinine. In these respects, at least, the resemblance to *vivax* malaria continues.

HUMAN MALARIA

As mentioned earlier in this paper, non-circulating parasites in the human malarias have been deduced from chemotherapeutic and subinoculation studies. The actual demonstration of these forms in the avian malarias provided a dramatic stimulus to the acceptance of such a concept for simian and human malarias, as it is easier to believe that there is a basic similarity in the life cycles of the various species than that they are widely divergent. Most malariologists now accept as

their working hypothesis the following ideas: (1) that in human malarias initiated by sporozoites there are one or more generations of development in non-circulating and probably non-erythrocytic cells; and (2) that in certain of the malarias, notably *vivax* and probably quartan, non-circulating forms are responsible for the persistence of the infection and for that type of relapse which follows therapy sufficient to eradicate erythrocytic parasites. Huff (1947) has presented, in considerable detail, the evidence for such a working hypothesis. Such an hypothesis does not specify where the early pre-erythrocytic development occurs, nor what the forms look like, nor does it specify whether or not the forms responsible for relapse are lineal descendants of any of the pre-erythrocytic forms which give rise to the early erythrocytic attacks. The persistent forms may be continually or sporadically reproducing under conditions whereby attrition virtually balances multiplication. It is possible that certain sporozoites after entering the body remain in a resting stage for weeks or months, as suggested by Shute (1946), or, after going through a portion of their pre-erythrocytic cycle, they enter such a resting stage.

Fairley et al (1947) have shown that subinoculations of blood within the first 30 minutes after the introduction of sporozoites will initiate malaria in susceptible recipients and that this is followed by a negative phase during which subinoculations are uniformly unsuccessful until about the 7th day in *falciparum* and about the 9th day in *vivax* infections. Many studies, including our own (Coatney, Cooper, Ruhe et al, 1948), have shown that quinine given during the first 6 days does not even delay the appearance of patent erythrocytic infection, which it would most certainly do if the erythrocytic cycle had already been initiated. Subinoculation studies by Fairley et al (1947) have shown that neither quinacrine nor chloroquine delays the appearance of circulating parasites either and, therefore, these drugs, too, have no inhibitory action on the early pre-erythrocytic stages. This provides the general basis for our belief that there is a non-circulating stage during the first 6 days or so of *vivax* and *falciparum* malaria which is relatively unaffected by quinine, quinacrine or chloroquine.

Sporozoite-induced *vivax* malaria is characterized, as is well known, by repeated relapses, sometimes continuing for several years after the original infection. With some strains, notably the St. Elizabeth, the interval between the first and second attacks may be many months. It is impossible for us to account for this long interval on the basis of immunity to erythrocytic parasites (Cooper, Coatney and Ruhe, 1947). It is more likely that it represents a period when the fixed-tissue reservoir is not casting erythrocyte-invading forms into the circulation. Evidence against persisting erythrocytic parasites being responsible for these repeated relapses is provided by the fact that dosage regimens of drug sufficient to completely eradicate blood-induced infections will not eradicate sporozoite-induced *vivax* malaria (Shannon, 1946; Shannon et al, 1948). Furthermore, Cooper, Ruhe and Coatney (unpublished) have shown that massive subinoculations carried out during latency in St. Elizabeth strain *vivax* malaria do not result in the transmission of infection to susceptible recipients.

P. falciparum almost certainly has pre-erythrocytic stages, also, but since most strains fail to relapse, we are led to believe either that there are no persistent non-circulating parasites or if such forms are present, they are vulnerable to the conventional antimalarials or to the immune mechanisms. It is probable that most

relapses following therapy of *falciparum* malaria result from the persistence of erythrocytic parasites, as widely variant thresholds of response to conventional antimalarials have been demonstrated (Earle et al, 1948).

Representatives of most of the chemical groups discussed under avian malaria have been tested in man and the evidence for action against the hypothetical exoerythrocytic stages will be presented briefly.

1. *Quinoline derivatives.* In *vivax* malaria, at least, quinine and chloroquine, as well as the acridine compound quinacrine, show no significant activity against E-E stages. They will not prevent infections when given in massive dosage for from 1 to 3 weeks after exposure and will not cure established infections.

The only unequivocal demonstrations of causal prophylactic and curative action in man have been among the 8-aminoquinolines (e.g., pamaquine). It will be recalled that in the birds these compounds showed a low order of activity against exoerythrocytic forms although some (see Wiselogle, 1946) were 50 to 100 times as effective as quinine against erythrocytic parasites.

James, Nicol and Shute, in 1931, began a series of prophylactic experiments with pamaquine, which eventually included some 50 volunteers; the results were summarized in a League of Nations report (1933). These studies showed that pamaquine, in dosage of 60 to 80 milligrams of base daily for 6 days after exposure, would permanently prevent *P. falciparum* and would protect a high percentage of subjects against *P. vivax*. Although the results with respect to *P. vivax* were quite different from those obtainable with any other drug, they were not followed up for over 10 years, doubtless because the dosage of pamaquine needed was too dangerous for any possible practical application. However, during recent years James, Nicol and Shute's results have been amply confirmed by Feldman et al (1947), by Fairley et al (1947) and by Jones, Craige et al (1948).

Three other 8-aminoquinolines, SN 1452, SN 11,191 and pentaquine (SN 13,276), have also been shown by Jones, Craige et al (1948) to be prophylactic against Chesson strain *vivax* malaria. Even the patients in whom complete prophylaxis was not obtained exhibit a modified infection, suggesting a partial prophylactic effect.

All of these conclusive prophylactic studies were in experimentally infected patients and the effective dosages were in a toxic range impractical for field use.

The 8-aminoquinolines have more practical importance with respect to cure of *vivax* malaria. Before the war several investigators reported studies indicating that pamaquine in sufficient dosage and in combination with quinine would reduce the relapse rate in *vivax* malaria (Sinton and Bird, 1928; Sinton, Smith and Pottinger, 1930; Manifold, 1931; Jarvis, 1932, 1932a; Dixon, 1933). These early reports have been confirmed in recent years both in natural infections (Kelleher and Thompson, 1945; Most et al, 1946) and in controlled experimental infections (Berliner, Taggart et al, 1946; Craige et al, 1947; Berliner, Earle et al, 1948; Ruhe et al, unpublished). In the experimental infections higher dosages were required for cure, probably because of the heavy sporozoite inocula and because treatment was begun early in the course of the disease.

Great effort has been expended in attempts to find an 8-aminoquinoline which is curative of *vivax* malaria but less toxic than pamaquine. Of the many analogues tried in man, at least five (SN 9972, SN 13,274, SN 13,276, SN 13,429 and DR

15,302) have been shown to be curative (Alving, Craige et al, 1948; Alving, Pullman et al, 1948). Two of these, SN 13,274 (isopentaquine) and SN 13,276 (pentaquine), appear definitely superior to pamaquine.

It is of interest, also, although not unexpected, that the 8-aminoquinolines are curative of *vivax* malaria when given during latency. The St. Elizabeth strain of *P. vivax,* with its period of 6 to 9 months of latency between early and late periods of activity, afforded an ideal opportunity for testing this possibility; pentaquine plus quinine given to five patients 4 months after exposure resulted in complete cures while quinine-treated controls all had relapses at 7 to 10 months (Coatney and Cooper, unpublished).

2. *Sulfonamides.* Several sulfonamides have been studied in human malaria. As early as 1939, Sinton, Hutton and Shute concluded that proseptasine (N^4-benzylsulfanilamide) was a true causal prophylactic against *P. falciparum* but no further work with this compound is known to us.

Sulfadiazine, one of the most potent prophylactic agents in *gallinaceum* malaria, has failed in our hands (Coatney, Cooper, Young and McLendon, 1947; Coatney, Cooper, Young, Burgess and Smarr, 1947) as a prophylactic in *falciparum* and *vivax* malarias. In the former, only moderate delay was obtained when dosages of 12 grams daily were given for 2 days after exposure. In *vivax* malaria, 3 grams daily for 42 days after exposure suppressed the early attacks but did not prevent the infections from becoming established. Sulfadiazine has likewise proved ineffective for the cure of *vivax* malaria, as shown by Coggeshall, Martin and Bates (1945).

Metachloridine, the most promising of the metanilamides, has also failed as a causal prophylactic and curative agent in *vivax* malaria. Alving (1946) has shown that Chesson strain *vivax* infections were not prevented by metachloridine continued for either 6 or 20 days after exposure. In our studies at Atlanta we were unable to obtain cure of the St. Elizabeth strain of *P. vivax* by 12-day courses of the drug, whether given alone or with quinine (Cooper, Ruhe, Coatney and Josephson, unpublished).

3. *Naphthoquinones.* Packer and Watson (1946) tested three naphthoquinones, SN 3529, SN 5090 and SN 8557, as prophylactic agents against McCoy strain *vivax* malaria and found that none of them prevented infections. SN 8557 also failed to prevent Costa strain *falciparum* malaria. Ruhe et al (unpublished) have tested this last-named drug as a curative agent against St. Elizabeth strain *vivax* malaria in dosage of 1.0 gram daily for 12 days, after a 6-day course of quinine. Relapses occurred in two of three subjects. It should be pointed out, however, that this dosage was, in milligrams per kilogram per day, only one-tenth that used by Gingrich and his group (Gingrich, 1946; Gingrich et al, 1947) in the canary.

4. *Biguanides.* The two biguanides (M.4430 and M.4888) found by Curd, Davey and Rose (1945) to be active against E-E forms in avian malaria have been tested in man. M.4888 (paludrine), which had the broader range of prophylactic activity against the avian malarias, has been shown by Fairley et al (1946) to have extremely interesting properties in man. Infection by *P. falciparum* was permanently prevented by single doses of paludrine given during the first 5 days after exposure. Relatively large doses, e.g., 1.0 gram, were necessary if given on the day of exposure but small doses, e.g., 10 to 100 milligrams, were sufficient during the period from 48 to 120 hours after exposure. Sustained daily doses during this early period were

uniformly effective. Subinoculations on day 7 were positive in subjects receiving quinine, quinacrine, chloroquine or sulfadiazine (where prevention of *falciparum* malaria is attributed to action on erythrocytic parasites), but were negative in subjects whose malaria was prevented by paludrine. *P. vivax* was delayed but not prevented by paludrine, even in high sustained dosages, but, even here, subinoculations on days 9 to 11 were negative.

We believe that these well-conceived studies by Fairley and his group come very close to proving the activity of paludrine against the actively developing pre-erythrocytic stages in both *falciparum* and *vivax* malaria. In the latter infection, the activity would be assumed to be incomplete either in the sense of producing only temporary arrest of development, as suggested by Fairley, or to a complete lack of effect on some coexistent stage. A possible alternative explanation, one considered by Fairley et al (1947, p. 669), is that paludrine, or an active metabolic product, might act by persistence in the blood into the period of erythrocytic schizogony. Under such conditions it could conceivably be so active against erythrocytic forms that they would promptly be rendered non-viable, and thereby account for negative subinoculation. Against this, is a statement by Fairley et al, in another context (1946, p. 138), that they had secured positive subinoculations on the third day of therapy of acute attacks despite 1.0 gram of paludrine daily. Blood levels of paludrine do not satisfactorily answer the question of persistence of effect. Despite the fact that levels decline rather rapidly after single doses, there is evidence (Hawking, 1947), as brought out under avian malarias, that paludrine as measured is not the active principle but that a metabolic product formed in the animal host produces the antimalarial effect. This metabolic product may remain in the body longer than now realized.

In our own studies (Coatney and Cooper, unpublished) continued administration of paludrine for as long as one year was followed by prompt appearance of *P. vivax*. Furthermore, paludrine in high dosages does not cure *vivax* malaria (Maegraith, Adams, King, Tottey et al, 1946; Maegraith, Adams, King Rigby et al, 1946; Fairley et al, 1946; Johnstone, 1946, 1947; Jones, Pullman et al, 1948; Coatney and Cooper unpublished), a fact which is in keeping with its inability to prevent such infections.

In practical application, paludrine does nothing that cannot be done with chloroquine but there are strong indications that its mode of action is quite different and that it does act against non-circulating stages immediately preceding the erythrocytic cycle. The only loophole in the proof for this is the possibility of a highly efficient metabolic product of paludrine persisting into the period of erythrocytic schizogony. No tolerable dosage of paludrine will destroy the forms of *P. vivax* responsible for relapse.

SUMMARY

Chemotherapeutic effects attributable to action on exoerythrocytic stages have been discussed with reference to avian malarias where E-E forms have actually been demonstrated and in simian and human malarias where presence has been deduced from indirect evidence.

In avian malarias, certain sulfonamides, naphthoquinones, biguanides, acridones, *s*-triazines and one other compound, a pyrimidine, exert causal prophylactic effects

attributable to action against pre-erythrocytic stages. However, only one drug, a naphthoquinone, has so far been found capable of curing an established infection where exoerythrocytic forms are known to occur.

In the monkey, only *Plasmodium cynomolgi* has been studied under conditions favorable to obtaining data on exoerythrocytic forms and their response to drugs. The data now at hand show that representative 8-aminoquinolines with or without concomitant quinine will cure established sporozoite-induced *cynomolgi* infections.

In human malarias, we find only two groups of drugs, the 8-aminoquinolines and the biguanides, which show evidence of activity against hypothetical exoerythrocytic stages. The 8-aminoquinolines are prophylactic and curative against both *P. falciparum* and *P. vivax*. Paludrine, the most thoroughly studied biguanide, is apparently prophylactic against *P. falciparum* and partially prophylactic against *P. vivax*, but it will not cure an established *vivax* infection.

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SYMPOSIUM ON EXOERYTHROCYTIC FORMS OF MALARIAL PARASITES

IV. THE CHEMOTHERAPY AND IMMUNOLOGY OF PRE-ERYTHROCYTIC STAGES IN AVIAN MALARIA

FREDERICK COULSTON[1] AND CLAY G. HUFF[2]

With the demonstration of the development of *Plasmodium gallinaceum* from sporozoite to erythrocytic trophozoite and the localization and distribution of the cryptozoites and metacryptozoites, it was possible to consider immunological and chemotherapeutic aspects of the pre-erythrocytic stages (Huff and Coulston 1944; Coulston, Cantrell and Huff, 1945). Similar but less complete studies were done on the pre-erythrocytic development of *P. relictum, P. cathemerium,* and *P. lophurae,* (Huff and Coulston, 1946; Coulston and Huff, 1947; Huff, Coulston, Laird, and Porter, 1947; Huff and Coulston, 1943, CMR Progress Report, 12). In 1943 Coulston and Huff (CMR Progress Report, 12) devised methods whereby the *in vivo* effects of drugs could be observed cytologically on the pre-erythrocytic stages of *P. gallinaceum.* We are presenting here the results of such studies together with an attempt to relate these results to our knowledge of the immune relations between pre-erythrocytic, erythrocytic and phanerozoic stages of certain malarial parasites and their avian hosts.

MATERIALS AND METHODS

Young and adult white leghorn chickens were used throughout these experiments for the study of the development of the pre-erythrocytic stages of *P. gallinaceum* (*8A* strain). *Aedes aegypti* mosquitoes were infected by feeding them upon severely parasitized chickens. Sporozoites were obtained by dissecting out the salivary glands of the infected mosquitoes.

For complete details of the histological and parasitological methods employed the reader is referred to the paper by Huff and Coulston (1944) in which these methods are described in detail.

EXPERIMENTAL

a. *Causal Prophylactic Drugs and Pre-erythrocytic Stages*

Cryptozoites. At the time this work was started, it was known that certain sulfonamides acted prophylactically against sporozoite induced *P. gallinaceum* infections. Consequently, sulfadiazine, sulfamerazine, and sulfapyrazine as well as a metanilamide (SN-11439)* and a napthoquinone (SN-12,320)† were fed to chickens at percentages in the diet of from 0.05 to 4.0%. Blood levels of these drugs were obtained many times the amount necessary to prevent blood infections produced

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* 2-Metanilamido-5-chloropyrimidine.

† 2-[3-(decahydro-2 naphthyl) propyl]-3 hydroxy-1,4-naphthoquinona.

by either infected mosquito bites or by the intravenous injection of sporozoites. Despite this, cryptozoite development occurred in both the skin and the spleen. Morphologically, the cryptozoites appeared normal in most instances and only occasionally were abnormal parasites observed. Such abnormal parasites were characterized by large vacuoles, irregular in size (see Figs. 1 and 2; Plate I). It will be recalled that the normal appearing schizont stained by the Maximow method became highly vacuolated as it approached segmentation (36 hours) and that these vacuoles were very regular in character. Badly damaged cryptozoites were most often seen in animals treated with sulfadiazine and naphthoquinone, and to a lesser extent in those treated with metanilamide. However, it should be remembered that the great majority of these cryptozoites were not only normal in appearance, but also there was no apparent reduction in the numbers of cryptozoites in chickens fed such drug diets.

Metacryptozoites. The effect of the causal prophylactic drugs against the metacryptozoites was very evident. Cytological changes were characterized by the presence of large vacuoles in the cytoplasm and the lack of development of the metacryptozoites (see Figs, 4 and 5, Plate I). Untreated parasites at 72 hours have usually attained their maximum growth but under the effect of the drugs most of them were smaller than normal metacryptozoites. Many of the parasites at this stage of development appeared to be undergoing a process of dissolution and with the poor staining characteristic of debris.

Another important factor which indicated why these drugs prevented parasitemia in the chicken was the appreciable reduction observed in numbers of metacryptozoites. Although this reduction in number of parasites was subject to experimental error, it was felt that the methods were standardized sufficiently to minimize this criticism. Therefore, it could be possible that the drugs acted upon many of the cryptozoic merozoites as they were released into the tissue spaces and injured them so that they died when they were phagocytized. This important point is worthy of reinvestigation. The fact remains that a serious reduction in the number of parasites occurred during the period of the second generation which, coupled with the marked cytological damage done by the drugs, placed the parasite at a great disadvantage.

These results are also in line with unpublished data indicating that when birds were taken off the drug diet, 72 hours after sporozoite inoculation, many of them became infected. On the other hand, if the drug was continued for a total of 120 hours none of the chickens became infected. Occasionally, normal metacryptozoic segmenters were found in the wing skin, liver, and spleen 72 hours after sporozoite inoculation in birds on drug-diets.

b. *Suppressive Drugs and Pre-erythrocytic Stages*

Suppressive drugs such as quinine, quinacrine, chloroquine, pamaquine, and pentaquine were studied in a manner similar to the prophylactic drugs. Morphological changes upon the cryptozoites and metacryptozoites of *P. gallinaceum* (see Plate I; Figs. 4, 5, 7) were observed with all of these drugs. In no case was the number of parasites affected as great as with the prophylactic drugs such as sulfadiazine. Also, no discernible reduction in the frequency of metacryptozoites occurred with the suppressive drugs. Except for frequent abnormal schizonts, they appeared similar to the pre-erythrocytic stages observed in the controls.

Metacryptozoites developed normally and metacryptozoic merozoites were produced even during continuous drug therapy and occasionally a flooding effect occurred. This was particularly true if the level of drug in the diet was not too high. In such chickens, small merozoites were seen on or in erythrocytes and it could be presumed that the suppressive drug became effective at this point in the infection.

DISCUSSION

1. *Theoretical Relationship Between Pre-erythrocytic Stages and Phanerozoites*

In a discussion of this kind, it is well to consider the differences between blood induced malaria infections as compared to sporozoite induced infections. The classic discovery by James and Tate (1938) that certain developmental stages of *P. gallinaceum* in chickens appeared outside of erythrocytes, to which they gave the name *exoerythrocytic stages,* gave stimulus to the study of this new phase of malariology. It will be recalled that previously Huff and Bloom (1935) observed that certain stages of *P. elongatum* in the canary lived in the blood and blood-forming cells and that the parasites of the hemoglobin-free lymphoid and myeloid cells were unpigmented. Previously Raffaele (1934) had found with the same species of avian malaria that the parasites preferred young erythrocytes, but there is still some question as to the exact nature of what was described. However, in 1936 Raffaele made an outstanding contribution to malariology when he observed a few large exoerythrocytic stages in sporozoite induced infections of *P. relictum* at the time of parasitemia. This discovery was confirmed and extended to other species of avian malaria (Kikuth and Mudrow, 1937; James and Tate, 1938; Coulston, 1939; Manwell and Goldstein, 1938a, b; for a review of this subject see Porter and Huff, 1940; Huff and Coulston, 1944; Huff, 1947).

It is now recognized that the events following blood-induced infections of *P. gallinaceum* differ from those occurring from sporozoite-induced infections. When malaria is produced by the inoculation of parasitized erythrocytes, exoerythrocytic stages (phanerozoites, Huff and Coulston, 1946) appear late in the infection, usually reaching a maximum in numbers during the third or fourth week. The phanerozoic stages gradually increase in numbers beginning at about the time of the crisis of the parasitemia. As pointed out by Huff and Coulston (1946), this difference in point of time of the maximum number of parasitized erythrocytes and the number of phanerozoites is of great theoretical importance, for it demonstrates an almost complete independence of the two components of the blood-induced, avian malarial life cycle. In fact, it is possible to consider these two components of the life cycle as two separate cycles and from a theoretical point of view this has enabled us to interpret more clearly the relationships between exoerythrocytic stages and parasitemia. That these two cycles are related was demonstrated by Coulston and Manwell (1941) for *P. circumflexum* and by Downes (1947) for *P. gallinaceum* when they injected single parasitized erythrocytes into birds which developed both parasitemia and phanerozoites. Furthermore, tissue culture and chick embryo passage of *P. gallinaceum*-infected tissue rich in exoerythrocytic stages, has revealed that the cycles may exist separately for a long time in continuous passage (Haas, Wilcox, Laird, Ewing, and Coleman, 1947; Lewert, 1948).

The separation of the life cycle of blood induced infections into two component parts enables one to better understand the sequence of events following sporozoite-

induced avian malaria. Birds inoculated with sporozoites develop cryptozoic and metacryptozoic forms *prior* to parasitemia. Erythrocytes may become infected as early as 72 hours after sporozoite inoculation in *P. gallinaceum,* but usually parasitemia occurs between the 5th and 8th day. At this point in the pre-erythrocytic cycle, a climax is reached in which metacryptozoic merozoites shift to erythrocytes, and a patent parasitemia develops. This normal transition of infection from the tissue stages to the erythrocytes may be considered the most important event of the life-history of the malarial parasite in the avian host, since to complete the cycle, gametocytes must be made available to the mosquito. As demonstrated by Huff and Coulston (1946) and Coulston and Huff (1947), it is at this transitional point that the "tissue-blood barrier" exists. These differences between the development of exoerythrocytic stages derived from the sporozoite as contrasted with parasitized erythrocytes represent the considerations upon which any theory relating to natural and acquired immunity or chemotherapy must be based.

2. *Natural Immunity of Various Hosts to the Pre-erythrocytic Stages of P. gallinaceum*

A study of the behavior of pre-erythrocytic stages of *P. gallinaceum* in hosts possessing varying degrees of natural and acquired immunity to the disease was the next step. The basic studies necessary to such a comparative study had already been carried out on *P. gallinaceum* (Huff and Coulston, 1944; Coulston and Huff, 1946).

a. *Chicken* (Complete susceptibility). Huff and Coulston (1944) demonstrated conclusively that the normal parasitemia of the chicken by *P. gallinaceum* is initiated by the injection of sporozoites by or from a mosquito and starts as an infection in the macrophages of the skin and spleen and probably the endothelial cells of the blood vessels. In these cells, the sporozoites develop into cryptozoites, the name applied to the first generation pre-erythrocytic forms (Huff, Coulston, and Cantrell, 1943). At approximately 40 hours, cryptozoic merozoites enter macrophages and other cells of the lymphoid-macrophage system as well as true endothelial cells, thus initiating the metacryptozoic generation (Huff and Coulston, 1944). A few parasites may enter the blood at the end of this phase of the tissue parasitism, but these are very few in number. As already mentioned, patent parasitemia occurs definitely at the climax of the metacryptozoite development with a flooding of merozoites from the tissues into the circulating blood. With the increase in erythrocytic parasites, a typical blood infection is initiated which may be followed by the death or recovery of the bird. Phanerozoites by definition are present at this crisis although, admittedly, it is impossible as yet to separate the late metacryptozoites from the early phanerozoites. However, Huff and Coulston (1944) did distinguish between macro- and micromerozoite types of metacryptozoites indicating that, as the pre-erythrocytic tissue infection progressed, there is a shift in numbers from the macro- to the micromerozoite metacryptozoite. At the time of the flooding effect, the preponderance of micromerozoite metacryptozoic stages probably consists of early phanerozoites. This, then, is the normal development of *P. gallinaceum* in an entirely susceptible host, the chicken.

b. *Canary* (Complete immunity). On the other extreme of susceptibility, the canary appears to be completely immune to *P. gallinaceum* whether infection is at-

tempted by the inoculation of erythrocytic parasites or sporozoites. The natural immunity of this host appears to be directed against the sporozoite in the natural infections, since evidence has not been obtained indicating that they ever become established as cryptozoites. Likewise, injections of large numbers of parasitized chicken erythrocytes are followed by a rapid clearing of the circulation of the canary and no parasitemia is produced.

c. *Duck* (Partial immunity). The susceptibility of the duck to *P. gallinaceum* presents a most interesting example of the partial, natural immunity which led directly to the proposal of the term, tissue-blood barrier. The natural immunity of the duck places it midway between the completely susceptible host, the chicken, and the insusceptible host, the canary. Sporozoites of *P. gallinaceum* inoculated into the skin of the duck produce abundant and normal appearing cryptozoites and metacryptozoites but no visible parasitemia occurs with ordinary laboratory inoculums. Huff and Coulston (1946) did observe one parasitized erythrocyte in a small duck following the intravenous inoculation of heavily infected salivary glands from 200 mosquitoes. Even after repeated injections of large numbers of sporozoites, however, patent parasitemia could not be demonstrated on other occasions. When infected chicken blood was inoculated intravenously into ducks, there resulted a slight parasitemia which persisted for several days. However, subpatent infections could be produced in many ducks after the injection of either infected erythrocytes or sporozoites of *P. gallinaceum*. Some of these subpatent infections persisted for at least 8 months, as demonstrated by subinoculating the infected duck's blood into normal chickens. A measure of the low grade infection in the blood of the duck was indicated by the fact that not all sub-inoculated chickens, injected with the blood of infected ducks, developed frank infections. Often only one of two chickens receiving an aliquot of infected duck blood became infected. These results have at least two possible explanations: (a) the parasites are present in such low numbers in the erythrocytes that they cannot be observed easily by visual means; or (b) a tissue parasitism may exist which is transmitted through the blood by circulating phanerzoic merozoites either free or in leucocytes and cells of the lymphoid-macrophage system.

d. *Goose, Turkey, and Guinea Fowl* (Low-grade partial immunity). When sporozoites of *P. gallinaceum* derived from mosquitoes fed on infected chickens are inoculated into the goose or turkey, mild transient parasitemia is produced and the infection quickly becomes subpatent. Parasitized erythrocytes of *P. gallinaceum* from the chicken injected into these birds produce also a mild transient parasitemia. Therefore, the goose and turkey differ from the duck in that a slight parasitemia occurs following the inoculation of sporozoites. These results on the turkey are in accord with the observations of Corradetti (1942) who demonstrated that transient parasitemia followed the sporozoite inoculation of *P. gallinaceum*. In degree of natural immunity, it is possible then to place the goose and turkey between the duck and chicken in regard to degree of natural immunity to pre-erythrocytic and erythrocytic stages. The behavior of *P. gallinaceum* in guinea-fowl was essentially the same as in turkeys and geese.

Another example of varying degrees of natural immunity in the host to pre-erythrocytic versus erythrocytic stages is found in the experiments with *P. relictum* in canaries and pigeons (Huff and Coulston, 1946; Coulston and Huff, 1947).

3. *Tissue-blood Barrier*

The behavior of *P. gallinaceum* in different avian hosts indicated that some kind of barrier existed between the parasitism of the tissues and the parasitism of the blood. This barrier is part of the general phenomena which can be called natural and acquired immunity. The fact that ducks inoculated with sporozoites of *P. gallinaceum* developed no visible parasitemia, but had abundant and normal pre-erythrocytic stages, led to the introduction of the term, *tissue-blood barrier* (Huff and Coulston, 1946). This term simply calls attention to the processes of immunity which come into play during the transition from pre-erythrocytic to erythrocytic development. We do not imply that the barrier is a single one or that it is of a physical nature. The facts indicate that the transition from the tissue to the blood phase is one fraught with danger to the survival of the parasite and in this sense there is a barrier (or are barriers) to its successful growth in the host. As pointed out previously (Huff and Coulston, 1946), there are barriers at other points in the development of a full parasitemia from the inoculation of sporozoites into the host. The above discussion has called attention to the barrier that exists against the survival of sporozoites in certain hosts. Whether this barrier is similar to the one which prevents infection when trophozoites are introduced into the host is unknown.

Similarly, barriers no doubt exist between each pre-erythrocytic generation and the one following it. We know that some of the merozoites liberated by the mature schizont are engulfed by types of host cells in which they are incapable of living. Even in the macrophages which normally serve as host cells to the earlier pre-erythrocytic stages some parasites appear to be injured or killed (Huff, Coulston, Laird, and Porter, 1947).

4. *Interpretation of the Tissue-blood Barrier*

Since the tissue-blood barrier is but a reflection of the natural immunity of the host against parasitism, it is interesting to consider what evidence is available for interpreting the mechanism involved. It will be recalled that the main difference between sporozoite- and blood-induced infections consists in the time of appearance of exoerythrocytic stages in relation to the erythrocytic forms. This difference in *point of time* makes it possible to study this phenomenon.

As a starting point, the abnormal cycle, i.e., the stages both erythrocytic and exoerythrocytic following blood-induced infection, indicates the possibility that at least two antigen-antibody complexes may be implicated. In this type of infection it is readily observed that the blood and tissue stages are relatively independent of one another. The crisis of the erythrocytic stages occurs with no apparent effect upon the phanerzoites which are themselves increasing in numbers at this time. If the animal survives the erythrocytic infection, it may succumb to the phanerozoic infection which reaches its climax sometime during the 3rd to 4th week following blood-induced infection. Here again, the animal either dies or recovers in which case there is a diminution in the numbers of phanerozoites.

The normal cycle following sporozoite-induced infection reverses the sequence of events described above. Cryptozoites and metacryptozoites appear first and then the erythrocytic infection occurs. Since the invasion of the erythrocytes happens at the time of maximum numbers of metacryptozoites, it is difficult to separate the

two parts of the cycle at this point; but it is known that there is a reduction in the numbers of metacryptozoic segmenters at this time which soon coincides with the crisis of the erythrocytic infection.

Although various, attractive hypotheses may be built up to explain the tissue-blood barrier on the basis of one, two, or even three antibodies the status of our knowledge is such that, at present, our energies should be directed more toward the planning and execution of experiments designed to yield the data necessary to an adequate explanation of the present findings.

5. *Acquired Immunity and Pre-erythrocytic Stages*

Most of the information regarding the question of acquired immunity in malaria concerns the fate of the erythrocytic stage. With this stage of the malaria growth cycle, it is well established that there is a strong immune response to superinfection with homologous and certain heterologous strains of species of avian, simian, or human malaria. Huff and Coulston (1946) demonstrated that chickens which have had injections of sporozoites and erythrocytic parasites and have developed an effective immunity to superinfection as demonstrated by lack of parasitemia, nevertheless permit the development of normal appearing cryptozoites and metacryptozoites. This indicates that the immunity to erythrocytic infection is not sufficient or of the proper kind to prevent the development of the pre-erythrocytic tissue stages.

It is of particular interest to point out that even though normal appearing metacryptozoic merozoites are produced in the immune animal when superinfected with homologous sporozoites, usually no parasitemia occurs. In other words, the immune normal host (chicken) reacts to sporozoites (*P. gallinaceum*) just as a normal duck responds to an inoculation of similar sporozoites, i.e., tissue stages develop without parasitemia. In both cases (normal and abnormal hosts) there is no successful flooding effect. Therefore, at least in avian malaria, the flooding effect or transfer of metacryptozoic merozoites to erythrocytes is one of the fundamental steps in the development of the parasite, rivaling in importance the injection of the sporozoite by the mosquito and the ingestion of gametocytes by the mosquito.

6. *General Consideration of the Chemotherapy of Pre-erythrocytic Stages*

It is interesting to consider the fact that during the presentation of this paper, three phenomena have been described which prevent a successful flooding effect of the metacryptozoic merozoites and their establishment in the erythrocytes. One of these is the true tissue-blood barrier which may be explained as part of the natural immunity of selected hosts (duck); the second is the acquired immunity developed by the normal host (chicken) against homologous erythrocytic parasites; and the third is the one in which a suppressive drug is capable of preventing the successful transfer of metacryptozoic merozoites to the erythrocyte.

In the earlier forms of pre-erythrocytic stages it can be demonstrated that metacryptozoites, *per se,* may also be injured by the natural and acquired immunity of the host and certain causal prophylactic drugs. But only in the canary, did any mechanism, immunological or chemotherapeutic, prevent the growth of the cryptozoites of *P. gallinaceum.* Summarily, chemotherapeutic agents may produce the same end result as various degrees of natural and acquired immunity.

Further, it should be mentioned that since all the drugs tested exhibited some

degree of action on the pre-erythrocytic stages, the indication is strong that a really good suppressive may in fact have prophylactic potentialities. It is not inconceivable, if quinine could be placed strategically in the host cells of the pre-erythrocytic stages, that this drug might act as aa causal prophylactic in the same sense as sulfadiazine.

The paper by Coatney and Cooper immediately preceding this one in the symposium deals in detail with the data on the relation of chemotherapy to exoerythrocytic stages of malaria. (See also Huff, 1947, 1948.) If an analogy could be made between the immunology and chemotherapy of pre-erythrocytic stages, we could theorize that the causal prophylactic drugs are accomplishing in susceptible hosts what natural immunity does in certain less susceptible hosts following sporozoite-induced inoculations. Suppressive drugs are effective against the parasite during the flooding effect thus preventing parasitemia in a manner not unlike the action of the tissue-blood carrier.

SUMMARY AND CONCLUSIONS

1. A method for studying the cytological effects of various antimalarial drugs upon the pre-erythrocytic stages of *Plasmodium gallinaceum* was devised.

2. By this method it was found that:

(a) Cryptozoites were able to grow in the macrophages of the skin in chickens fed sulfadiazine, sulfamerazine, sulfapyrazine, metanilamide, and naphthoquinone in dosages several times as great as was necessary to prevent parasitemia. The majority of such cryptozoites were normal in appearance. However, sulfadiazine, naphthoquinone, and to a lesser extent, metanilamide produced marked damage to some of them.

(b) The effects of the above-mentioned prophylactic drugs upon metacryptozoites were more marked. These consisted of the development of large vacuoles, the reduction in numbers of merozoites produced and the delay in development.

(c) The suppressive drugs, quinine, quinacrine, chloroquine, pamaquine, and pentaquine produced morphological changes in both cryptozoites and metacryptozoites but no appreciable reduction in the numbers of merozoites.

3. Comparisons are made between the type of cytological effects produced by chemotherapeutic agents and the natural and acquired immunity of the hosts. These appear to be essentially similar.

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EXPLANATION OF PLATE I

Cryptozoites and metacryptozoites of *P. gallinaceum* drawn from histological sections of the wing skin of chickens treated orally with drugs. Sporozoites from the infected salivary glands of *Aedes aegypti* were inoculated into the wing skin of the birds, which was then removed and fixed in Zenker-formalin and stained by Maximow's hematoxylin eosin-azur method. Since transitional fibroblasts and macrophages are difficult to distinguish from each other, all host cells are called mononuclear phagocytic connective tissue cells when it is impossible to identify the cell type. Magnification × 3000. Drawn by Mrs. Esther Bohlman Patterson.

FIG. 1. Cryptozoic schizont at 36 hours during treatment with sulfapyrazine. Note beginning of central vacuolation; otherwise normal.

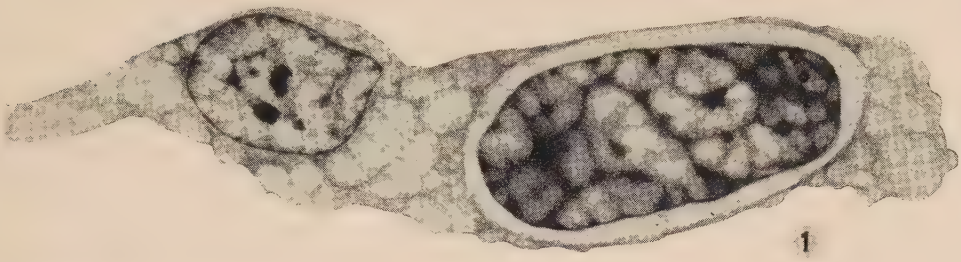
FIG. 2. 72-hour metacryptozoite, in a fibroblast, treated with sulfadiazine showing marked degeneration of the cytoplasm characterized by irregular vacuoles and roughness.

FIG. 3. A 39-hour cryptozoite indicating the early effect of sulfadiazine. Compare with Fig. 2.

FIGS. 4 AND 5. Degenerating metacryptozoites treated with quinacrine. At 72 hours; in macrophages.

FIG. 6. Dissolution of 72-hour, sulfadiazine treated, metacryptozoite. Compare with figures 1, 3, and 2 for sequence of degeneration.

FIG. 7. The effect of pamaquine upon a 70-hour metacryptozoite. Such parasites are not as numerous as they are during sulfadiazine therapy.



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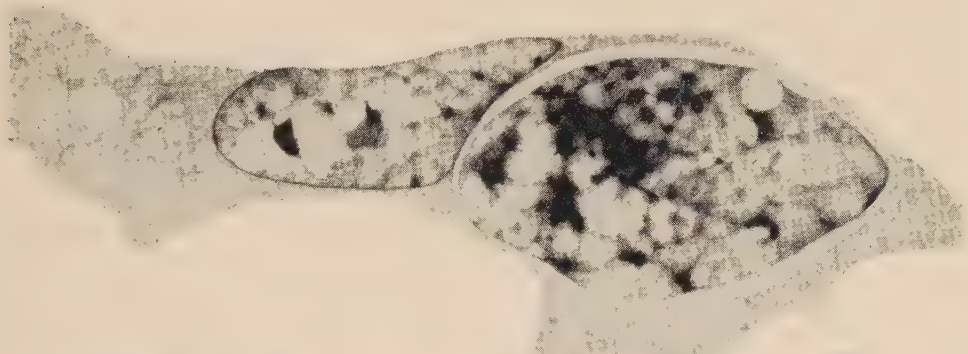

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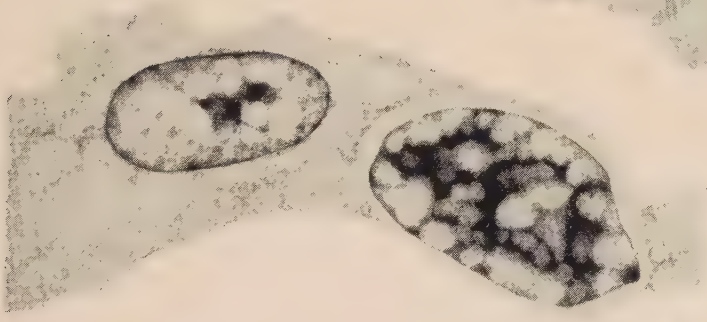

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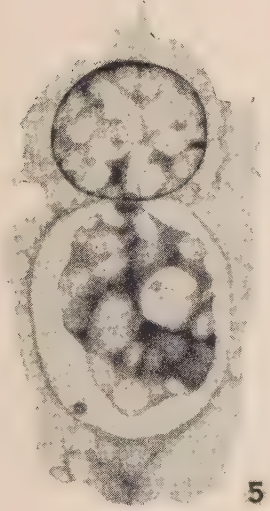

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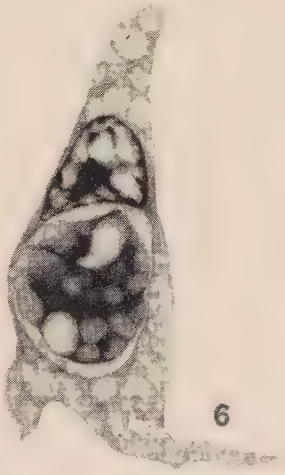

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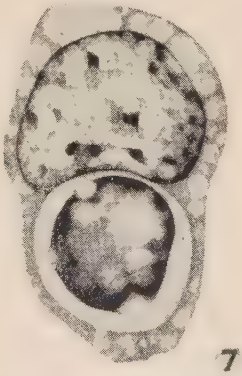

7

SYMPOSIUM ON EXOERYTHROCYTIC FORMS OF MALARIAL PARASITES

V. STUDIES IN TISSUE CULTURE OF EXOERYTHROCYTIC SCHIZOGONY IN AVIAN MALARIAL PARASITES

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The discovery of exoerythrocytic schizogony in certain avian malarias led very early to attempts at cultivation of the exoerythrocytic parasites *in vitro*. As early as 1935, in their study of the schizogony of *Plasmodium elongatum*, Huff and Bloom tried to grow the parasites in tissue cultures of the bone marrow of infected canaries. Parasites were not observed microscopically in these cultures, but evidence was obtained that they survived at least for short periods. By inoculation into normal birds the cultures were shown to contain viable parasites after 48 hours. Survival for such a short time would appear insignificant were it not for the fact that when cultures were made from tissues of birds infected with both *P. elongatum* and *P. cathemerium*, only the former was recovered after 48 hours. While the report does not demonstrate *in vitro* growth, it does indicate survival under conditions unfavorable to erythrocytic stages.

This study appears to be the only one reported on attempted cultivation of the *elongatum*-type exoerythrocytic schizonts, which develop in all cells of the erythropoietic series as well as in cells of the lymphoid-macrophage system. With the discovery of *gallinaceum*-type exoerythrocytic stages in *P. relictum* by Raffaele (1936), in *P. gallinaceum* by James and Tate (1937), and later in *P. cathemerium*, *P. circumflexum* and *P. lophurae*, efforts were instituted to demonstrate development of these parasites in connective tissue cells grown *in vitro*. In these species the exoerythrocytic stages develop almost exclusively in macrophages and capillary endothelium and are morphologically distinct from those reported in *P. elongatum*. This statement must be qualified with mention of the fact that in one individual chick embryo infected with *P. gallinaceum* Zuckerman (1946) found exoerythrocytic stages of the *elongatum* type.

Since macrophages grow readily in tissue culture, it appeared possible that these *gallinaceum*-type exoerythrocytic stages could be cultivated *in vitro*. The first attempts were those of Gavrilov, Bobkoff and Laurencin (1938). They cultivated fragments of tissue from chicks infected with *P. gallinaceum* in plasma and embryo extract. Cultures of bone marrow were incubated for seven days and then injected subcutaneously into a normal chick. The test bird became infected, with a prepatent period of eight days. In a similar fashion chicks were infected by intravenous and intramuscular inoculation of bone marrow cultivated for ten days. The long prepatent period, 11 days, indicated, however, that few parasites were present in the culture. Hanging-drop cultures of bone marrow were also infective after ten days, but efforts at subculture were unsuccessful. The authors divided and replanted cultures of the same series but obtained no evidence of persistence of infection. Normal bone marrow cultivated along-side the infected tissues did not become parasitized. Maitland cultures of surviving tissues (minced embryo and infected

bone marrow in serum-Tyrode solution) were not infective for test birds after four days' cultivation.

It is not clear whether the meager results obtained by these investigators reflect unsatisfactory methods or the use of poor sources of exoerythrocytic parasites. Their cultures were made from tissues of birds in the early stages of infection following inoculation of infected blood. If present, exoerythrocytic schizonts are certainly rare early in the course of such infections, and there is evidence that erythrocytic parasites give rise only sparsely to exoerythrocytic parasites in cultures of susceptible macrophages. These investigators themselves tried to infect cultures of normal tissues with blood from infected birds and failed consistently. In our laboratory we have similarly failed to infect macrophage cultures of various ages with erythrocytic parasites of *P. gallinaceum,* and it appears that special conditions are necessary for blood parasites to initiate exoerythrocytic development.

In an effort to test whether or not exoerythrocytic schizonts in canaries infected with *P. cathemerium* constituted a part of the life-cycle of the *Plasmodium,* Hegner and Wolfson (1939) made roller-tube cultures of the spleen, lung and brain of infected birds. Inoculated intramuscularly after eight days of incubation these cultures proved infective for normal canaries. The prepatent periods in the test birds, seven and eight days, indicated that considerable numbers of parasites were present in the cultures. It seemed unlikely that infection of the test birds resulted from surviving erythrocytic parasites, for the original tissues had been washed as free of blood as possible and the culture fluid had been replaced twice during the period of cultivation. At the time of these tests for infectivity the original cultures were subcultured, but no evidence of infection was obtained upon inoculation into normal birds seven days later. In a hanging-drop subculture fixed at the same time, however, exoerythrocytic schizonts were observed microscopically in macrophages.

While the studies summarized above appear to show some development of exoerythrocytic schizonts in tissue culture, it must be admitted that the results offered little for experimental exploitation. The investigations of Hawking (1945), however, revealed oustandingly successful methods for the study of exoerythrocytic parasites *in vitro.* With simple macrophage culture methods and exceptionally rich sources of exoerythrocytic parasites, Hawking demonstrated abundant development and very long survival of exoerythrocytic schizonts in culture.

Hawking used flask cultures and, in order to simplify the sampling of tissues for microscopic observation, he adopted the method of Jacoby (1944) whereby small cover slips are cemented with a plasma clot to the bottom of a Carrel flask. With this technique individual covers may be removed for study without affecting the rest of the culture, and it is possible to add normal tissues to growing infected cultures for the study of spread of infection. Most of the studies utilized fragments of spleen or buffy coat of the blood from chicks inoculated eight or nine days previously with sporozoites of *P. gallinaceum.* The tissues were cemented with a plasma clot to the cover slips in the flask. The fluid medium covering them consisted of Tyrode solution with 20 per cent normal chicken serum, two to six per cent extract of eight to ten-day chick embryos (three cc Tyrodes solution per embryo) and approximately three units of penicillin per cc.

Hawking reported that his medium contained 0.05 per cent Phenol Red as a pH indicator, but the report appears to err in this regard. We have found that this

concentration of Phenol Red reduced the pH of the medium to about six and prevented normal growth of the tissues. Approximately one one-hundredth of this amount gives a satisfactory color without affecting the pH of the medium. In some experiments Hawking adjusted the pH of the Tyrode solution to 7.6–7.8 before adding the other components of the medium.

In cultures of spleen or blood leucocytes from infected birds abundant growth of exoerythrocytic stages was observed. In one case normal chickens were infected by inoculation of material from a culture 89 days old. The abundance and time of appearance of parasites in the cultures depended on the intensity of infection in the source chickens, but in cultures made from heavily infected birds exoerythrocytic stages were found within two days. The parasites were always in cells identified as macrophages. No infection of fibroblasts was observed. In our laboratory we have confirmed this and also have been unable to recognize parasites in the epithelioid cells which become abundant in some cultures after two weeks or more. The greatest development of parasites was seen in cultures of spleen, blood leucocytes or bone marrow. Some growth was obtained in liver explants, but cultures of brain, lung and heart muscle failed to show infection.

In efforts to infect normal tissues from parasitized cultures Hawking introduced cover slips bearing uninfected macrophages into flasks containing abundant exoerythrocytic stages. Transfer of infection to these introduced tissues was demonstrated with a fair degree of regularity when the cultures were agitated on a rocking machine. In motionless flasks such transfer of infection was rare. Since macrophages migrate widely over the floor of a culture flask, these results do not necessarily indicate spread of parasites to cells from normal birds. The parasites did not become abundant in the new tissues, and an attempt to carry out a second passage was unsuccessful. In other experiments scrapings from infected cultures failed to infect cultures of normal tissues. Cultures containing exoerythrocytic parasites were infective on inoculation into normal chicks, but no evidence was obtained that parasites could invade erythrocytes introduced into positive cultures.

The exoerythrocytic stages in cultivated macrophages bore a general resemblance to those observed in capillary endothelium of infected chickens. In some cases they showed the merocyte formation reported by James and Tate (1938) in *in vivo* exoerythrocytic parasites. However, they differ in certain important respects from these phanerozoites, which appear late in infections induced by inoculation of parasitized blood or, according to Huff and Coulston (1944) five or six days after sporozoite inoculation. Unlike any of the types of exoerythrocytic schizonts seen in birds harboring *P. gallinaceum,* they usually showed a very prominent granule, presumably of volutin, near each nucleus, and most parasites had large vacuoles. Neither of these features is unique in the *in vitro* parasites, and their prominence may be merely an indication of slightly abnormal growth. The large schizonts were ovoid or discoid, rather than of the cylindrical shape usually seen in endothelium, and in this respect they resemble the majority of cryptozoites and metacryptozoites of Huff and Coulston (1944). This characteristic, of course, may have no fundamental significance but may merely reflect the shape of the host cell. In size and merozoite number the exoerythrocytic stages in culture are also more like cryptozoites than phanerozoites. The number of merozoites is very variable, but the extremely large

numbers, sometimes several hundred, seen in capillaries have not been reported *in vitro*. The merozoites are generally similar in size to those in cryptozoites.

For the reasons given it is not at all clear whether the exoerythrocytic schizonts observed in tissue cultures correspond to phanerozoites or to cryptozoites or metacryptozoites. In addition to their morphological similarities to cryptozoites, it should be noted that they are found in the same type of cell infected by cryptozoites and metacryptozoites. On the other hand, they were readily obtained in cultures from birds eight or nine days after sporozoite inoculation when, according to Huff and Coulston, phanerozoites predominate *in vivo*. Furthermore, Zuckerman (1946) obtained abundant growth of exoerythrocytic stages in cultures of the spleens of chick embryos inoculated with phanerozoites.

The general findings of Hawking have been confirmed by Zuckerman (1946) and by us. Zuckerman further found that the spleens of infected chick embryos gave cultures exceptionally rich in exoerythrocytic stages. We have used with success a macrophage culture method utilized by Zuckerman (1945) for other purposes. In this method leucocytes from citrated blood are washed in modified Tyrode solution (without calcium chloride) and suspended in serum for distribution in Carrel flasks. Zuckerman used flasks with the bottoms removed and large cover slips cemented in place of them. We find that Jacoby's technique of cementing small covers in the flasks is advantageous. In such cultures the leucocytes from infected chickens give rise to well-dispersed macrophages, many of which contain exoerythrocytic schizonts.

Using methods similar to those which were so successful with *P. gallinaceum,* Hawking (1946) has since reported growth of exoerythrocytic parasites of *P. relictum*. Birds were infected from *Culex pipiens,* and eight days later their spleens were explanted in the same medium used for *P. gallinaceum*. Despite the use of chicken serum, exoerythrocytic schizonts developed in the macrophages.

Tonkin and Hawking (1946) have similarly described growth of exoerythrocytic stages of *P. lophurae* in cultures made from the spleen of a turkey inoculated five days previously with sporozoites from *Aedes albopictus*. The parasites were similar morphologically to those of *P. gallinaceum* in culture.

In a series of cultures made from chickens at various intervals after sporozoite inoculation, Hawking (1945) obtained parasites in explants made as early as one hour after the injection of sporozoites. Only one of 21 cell colonies examined showed parasites microscopically. Negative results were obtained when cultures were made from tissues of chicks inoculated 15 minutes before, but only nine colonies were studied. These cultures were not infective when inoculated into normal chicks. Despite numerous variations of culture conditions, Hawking was unable to infect normal cultures with sporozoites from *Aedes aegypti*.

Previously, Rodhain, Gavrilov and Cowez (1940) had similarly failed to infect tissue cultures with sporozoites of *P. gallinaceum*. Cultures of chick embryo spleen, bone marrow or heart were studied as long as 22 days after addition of sporozoites and subinoculations were performed after eight days, but in no case was there evidence of growth or survival of the parasites.

Similarly, Paraense, Meyer and Menezes (1942) added sporozoites of *P. gallinaceum* to hanging-drop cultures of chick embryo spleen. No infections were found in four series of cultures inoculated with salivary glands and one series with stomachs

of the mosquitoes. Like Rodhain *et al* these authors found that the sporozoites degenerated rapidly and were not detectable after 24 hours. This is not surprizing in view of the findings in our laboratory (Porter, Laird and Dusseau, 1945) that at 37° C normal chicken serum rapidly destroyed the infectivity of sporozoites of *P. gallinaceum.*

In our laboratory numerous similar attempts to infect normal cultures with sporozoites have, with two possible exceptions, been equally unproductive. In one case sporozoites from *A. aegypti* were suspended in normal serum containing 4000 units per cc of penicillin and 20 mg per cc of streptomycin. This suspension was distributed to 11 macrophage cultures, seven to 28 days of age. Forty-eight hours later, scrapings and fluid from all cultures, heavily contaminated with yeasts, were inoculated intravenously to chicks. Only one culture showed evidence of infection, a single recipient chick out of four exhibiting blood parasites 15 days after inoculation. This culture was 21 days old at the time of sporozoite inoculation. In a later series, two cultures inoculated with sporozoites in serum and penicillin at the same time the leucocyte suspension was added (0 days old) and incubated for 48 hours produced infections in inoculated chicks with prepatent periods of seven to eleven days. In all, we have inoculated 60 cultures of various ages with sporozoites. At intervals from one to four days after addition of sporozoites they have given no indication of survival or growth of the parasites other than the dubious results cited here.

Despite the fact that sporozoites rapidly lose their infectivity in serum at 37° C, it must be emphasized that these results do not show that growth of exoerythrocytic schizonts occurred from the introduced sporozoites. Microscopic evidence or indirect evidence from much longer periods of cultivation is necessary to show that the findings here were not the result of survival of sporozoites in the cultures.

As to other lines of work with tissue cultures of exoerythrocytic stages, it must be admitted that up to the present there has been very little exploitation of the possibilities raised by these methods of cultivation. Hawking (1945) has made some study of the effects of the environment on exoerythrocytic parasites. He found that changes in oxygen tension, serum concentration and other conditions influenced growth, but he was unable to distinguish between effects on the growth of the infected cells and those exerted directly on the parasites.

While tissue cultures of macrophages are extremely complex systems, they do offer possibilities for far more control of the environment than is possible in the animal. At present it seems fair to say that only the basic methods have been developed and that no significant use has been made of those methods for the acquisition of new information about the exoerythrocytic stages of malarial parasites. Such information is to be expected from utilization of the available techniques.

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SYMPOSIUM ON EXOERYTHROCYTIC FORMS OF MALARIAL PARASITES

VI. RESPONSE OF EXOERYTHROCYTIC FORMS TO ALTERATIONS IN THE LIFE-CYCLE OF *PLASMODIUM GALLINACEUM**

Victor H. Haas, Aimee Wilcox, Raymond L. Laird,
Frances Moore Ewing, and Nell Coleman

PATTERNS OF PARASITE DEVELOPMENT

In discussing the exoerythrocytic forms of *Plasmodium gallinaceum* in 1938, James and Tate said that "there is great irregularity in the period of infection during which the exoerythrocytic stages are developed." It is now possible to discern a considerable regularity in exoerythrocytic development which was not apparent a decade ago. The present report delineates three general patterns of such development, representing different responses on the part of these forms to alterations in the life cycle of the parasite.

Pattern A.—The first of these patterns (Fig. 1) is that which occurs when *P.*

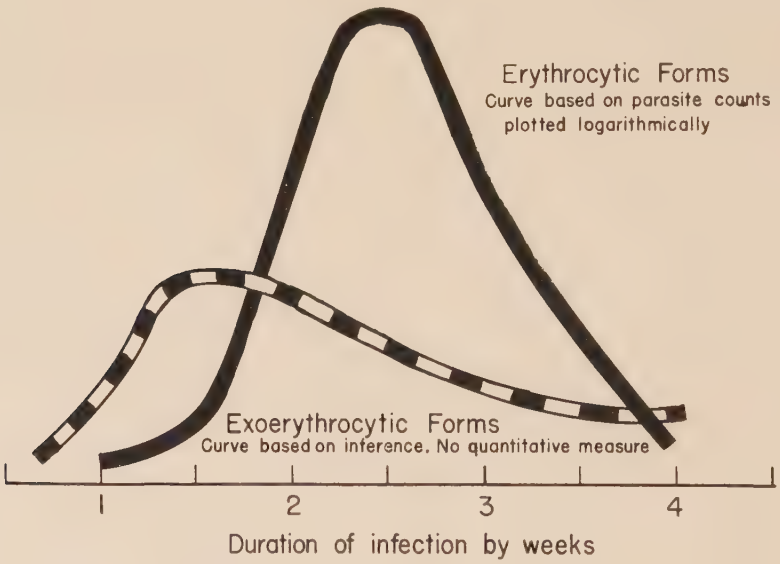

Fig. 1. Pattern A. Schematic representation of development of *P. gallinaceum* seen in chicks of alternating mosquito-chick series.

gallinaceum is transmitted alternately through mosquitoes and chicks. In view of the observations of Huff and Coulston (1944), it may be taken that the exoerythrocytic forms—using the term in its broad sense—here constitute the intermediate stages between sporozoites and erythrocytic forms. Their greatest prevalence pre-

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sumably occurs at about the time that the blood infection becomes patent. This pattern of development is the one which would be expected to occur in nature, the exoerythrocytic forms tending to subside as the erythrocytic infection increases, allowing the warm-blooded host to survive long enough to produce gametocytes. We have designated this pattern A.

In this "natural" type of infection where no blood-inoculations are interposed between mosquito-chick passages, the exoerythrocytic forms tend to be unobtrusive. In chicks routinely infected by inoculation with from 1 to 3 infected mosquitoes over a 3 year period, exoerythrocytic forms were found in only 1 out of 150 which died or were killed during the second and third weeks after inoculation. In a group especially inoculated for this study, using 1 infected mosquito per chick,[1] 1 out of 10 chicks was found to have exoerythrocytic forms in autopsy brain smears upon unusually extensive searching (Table 1). In another group inoculated for this

TABLE 1.—*Course of* Plasmodium gallinaceum *infection in chicks inoculated with sporozoites from alternating mosquito-chick transmission series. Each chick received equivalent of 1 whole infected mosquito intramuscularly (Coatney, Cooper, and Trembley, 1945). An example of parasite development pattern A. Unusually thorough search of brain smears was made at autopsy*

Chick number	Parasitized erythrocytes per 10,000 red blood cells on indicated post-inoculation day									
	7	8	9	10	11	13	14	16	17	18
1	0	0	0	5	80	2100	D–P	....	....	....
2	0	5	5	30	360	6100	....	4900	4600	D–P
3	0	0	10	20	80	6400	....	5200	D–P	....
4	0	5	0	10	120	5400	....	6700	D–P	....
5	0	0	0	20	250	6700	....	6700	D–P	....
6	0	0	0	5	20	1200	D–P	....	....	....
7	0	0	0	30	40	6800	D–P,E	....	....	....
8	0	5	20	90	630	4100	....	3500	D–P	....
9	0	0	0	20	80	5200	....	4300	D–P	....
10	0	0	0	60	1600	6800	....	5000	6200	D–P

D–P indicates death, pigmented erythrocytic parasites found in brain smear—No exoerythrocytic forms seen.

D–P,E indicates death, both pigmented erythrocytic parasites and exoerythrocytic forms found in brain smear.

study with from 1 to 3 infected mosquitoes each, no exoerythrocytic forms were found in brain smears made on birds killed or found dead on the third to the sixteenth days after inoculation in spite of the most thorough search (Table 2). Our failure to find exoerythrocytic forms in many of these chicks can not be taken to indicate a real absence of these stages, but it does demonstrate their scarcity under these conditions.

Huff and Coulston (1944) employed extremely heavy inoculations of sporozoites in order to get enough of these forms to follow their development microscopically. Under these circumstances, the concept of the developmental pattern of the exoerythrocytic forms is based on inference from the observations of Huff and Coulston, rather than on objective demonstration in the chicks in the usual mosquito-chick passage series, where small inocula are used.

Pattern B.—The second pattern, which we designate B, is seen in chicks infected by blood-inoculation. It is, therefore, a response to an alternation in the life-cycle of the parasite (Fig. 2). There is an acute stage, marked by heavy erythro-

[1] Throughout this report, chicks referred to were between 6 and 10 days old, unless otherwise noted. Nearly all sporozoite inoculations were by the "mosquito equivalent" method of Coatney, Cooper, and Trembley. (1945).

TABLE 2.—*Course of* P. gallinaceum *infection in chicks inoculated with sporozoites from alternating mosquito-chick series. Three chicks killed or dying daily examined for erythrocytic parasites in peripheral blood and exoerythrocytic forms in brains. Chicks examined on 3rd to 12th day received equivalent of 1 whole infected mosquito intramuscularly (Coatney, Cooper, and Trembley, 1945); others received 3 mosquitoes.*

An example of parasite development pattern A

3 chicks examined daily	Parasitized cells per 10,000 erythrocytes expressed as numerator, and type of parasites found in brain smears expressed as denominator, on indicatd post-inoculation days												
	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	0/0	0/0	0/0	0/0	0/0	0/0	1200/P*	0/0	5/P	10/P	3200/P	4500/P	2400/P	30/P
2	0/0	0/0	0/0	0/0	0/0	0/0	10/0	5/0	5/P	20/P	7500/P	3800/P	1200/P	80/P
3	0/0	0/0	0/0	0/0	0/0	0/0	10/0	30/0	10/0	....	2900/P	5200/P	5700/P	...

* P indicates pigmented erythrocytic forms found in brain smear—No exoerythrocytic forms seen.

cytic infection; many chicks die during this period, but in those which survive, exoerythrocytic forms become prevalent about the third week after inoculation, often with fatal results. This pattern was described by James (1939), and is apparent in the data shown in Table 3. The proportion of chicks falling in either category of this pattern is variable: if several million parasites are inoculated, practically all birds die of the acute erythrocytic infection; if only a few thousand parasites are given, a considerable number of chicks survive the acute stage and die later with the exoerythrocytic forms in their organs.

A strain of *P. gallinaceum* has been maintained in chick embryos by blood inoculation (Haas, Feldman, Ewing, 1945) for several years; after 193 serial pass-

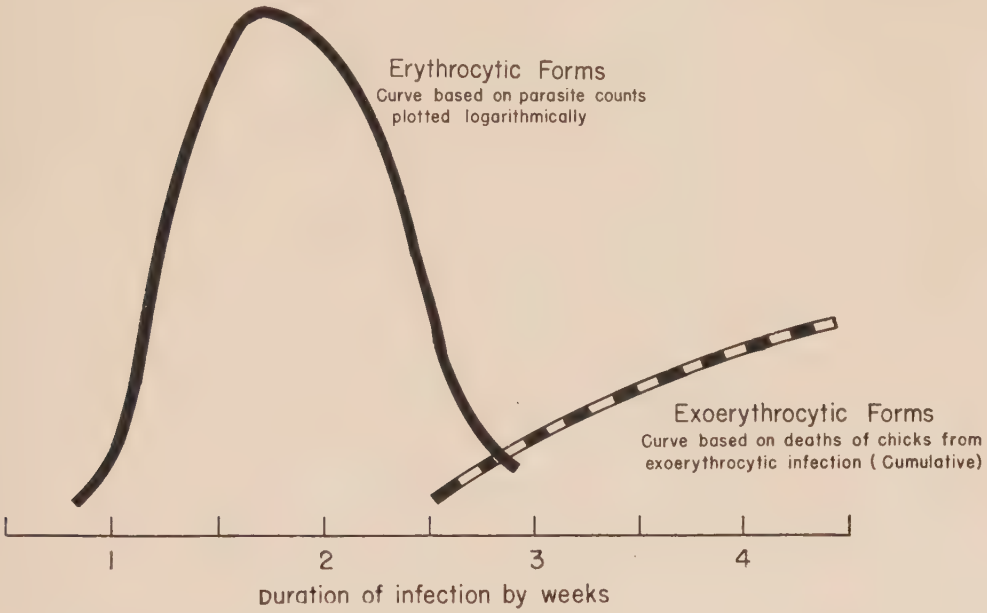

FIG. 2. Pattern B. Schematic representation of development of *P. gallinaceum* seen in chicks infected by blood inoculation.

ages, some of these parasites were transferred to chicks by blood inoculation: 8 chicks died of typical acute parasitemia, and one which survived this stage died of exoerythrocytic infection on the 18th day. Thus this strain, long maintained in embryos, still conforms to pattern B.

Pattern C.—The third pattern, representing another response of exoerythrocytic forms to unnatural alterations in the life-cycle, is the one with which this report is particularly concerned. We designate it pattern C (Fig. 3). It is marked by a predominance of exoerythrocytic forms, which attain sufficient prevalence early in the infection to kill the host before erythrocytic parasites become at all numerous, and in many cases before they are seen at all. The variety of circumstances under which this type of response occurs are discussed in the remainder of this report.

OCCURRENCE AND CHARACTERISTICS OF PARASITE DEVELOPMENT, PATTERN C

Brain-inoculated Chicks.—This predominantly exoerythrocytic type of *gallinaceum* infection—pattern C—is readily produced in chicks by using as an inoculum a

TABLE 3.—*Course of* Plasmodium gallinaceum *infection in selected chicks inoculated with parasitized blood, showing early predominance of erythrocytic parasites, and later prevalence of exoerythrocytic forms: An example of parasite development pattern B*

Chick no.	Parasitized erythrocytes per 10,000 red blood cells on indicated post-inoculation day																			
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	....	3300	4300	6600	7700	....	6000	....	6500	....	D–P*	....	....	....	....	....	....	....	....	....
2	....	0	0	....	10	....	1330	3000	8600	7600	....	D–P	....	....	....	....	....	....	....	....
3	....	20	....	1400	4600	....	D–P	....	....	....	....	....	....	....	....	....	....	....	....	....
4	....	1530	2100	6300	5900	4800	5300	....	D–P	....	....	....	....	....	....	....	....	....	....	....
5	....	8600	8000	D–P	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
6	3100	4500	7200	6100	4900	....	....	5500	5100	500	....	....	....	D–E†	....	....	....	....	....	....
7	....	2000	....	....	3900	....	5300	6100	6200	....	....	....	....	....	D–E	....	....	....	....	....
8	0	0	0	30	....	....	3900	....	....	....	....	....	200	100	....	....	....	....	....	D–E
9	....	0	....	30	30	....	2100	....	6100	....	5200	4700	....	50	....	10	....	....	D–E	....
10	30	....	....	360	....	....	....	7500	....	630	....	....	....	....	40	....	D–E	....	....	....
11	....	5	....	0	....	....	200	....	2600	....	0	10	....	0	....	40	....	D–E	....	....

* D–P indicates death, pigmented erythrocytic parasites found in brain smear.
† D–E indicates death, exoerythrocytic parasites found in brain smear.

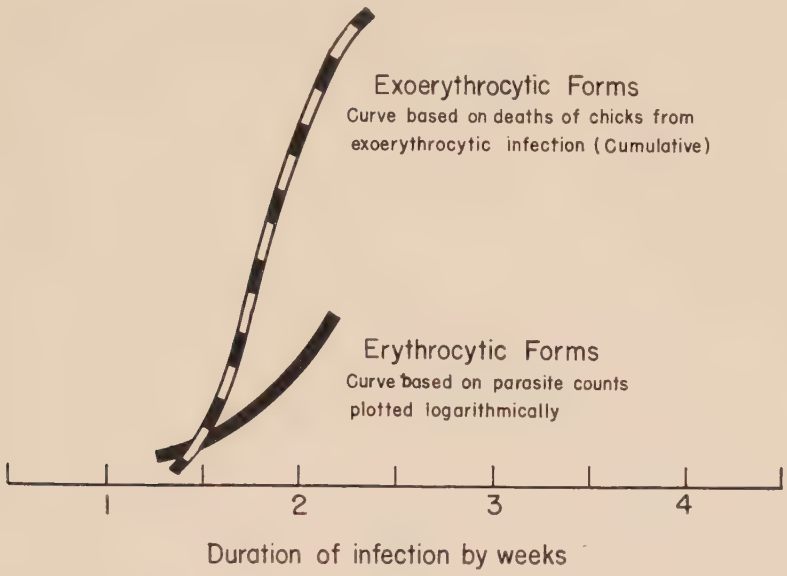

FIG. 3. Pattern C. Schematic representation of development of *P. gallinaceum* seen in chicks infected by brain inoculation and in other conditions.

saline emulsion of chick brains (usually 1 part brain to 9 parts saline) containing many exoerythrocytic forms (Haas, Wilcox, Davis, Ewing, 1946). Chicks inoculated intramuscularly with such material (dose: 0.5 cc) die on the 9th to 15th post-inoculation day, with numerous exoerythrocytic forms in their brains, but with very few parasites in the red blood cells, or with none at all so far as microscopial examination can detect (Table 4). Since our original report (Haas, Wilcox, Davis, Ewing, 1946) we have established this type of infection by serial passage and it now seems capable of indefinite maintenance in this form so long as the brain-emulsion inoculum is used.

Conversion to Pattern B.—Blood from chicks of this series inoculated intravenously into fresh chicks, produced at first a typical pattern C infection: low parasitemia and heavy exoerythrocytic infection. However, when this infection was carried serially by blood inoculation into other chicks, the exoerythrocytic predominance gradually disappeared (Table 5): the second blood-inoculation passage

TABLE 4.—*Course of* P. gallinaceum *infection in chicks inoculated with saline emulsion of brain infected with exoerythrocytic forms (1part brain to 9 parts saline. Dose: 0.5 cc intramuscularly): An example of parasite development, pattern C*

Chick no.	Parasitized erythrocytes per 10,000 red blood cells on indicated post-inoculation day					
	7	8	9	12	13	14
1	0	0	0	0	10	D–E*
2	0	0	0	0	0	D–E
3	0	0	130	D–E	..	....
4	0	0	0	D–E	..	....
5	0	0	5	70 D–E	..	....
6	0	0	0	0 D–E	..	....
7	0	0	0	70	30	D–E
8	0	0	0 D–E	..	..	....
9	0	0	0	5	0	D–E
10	0	0	0	0	0	D–E

* D–E indicates death, exoerythrocytic parasites found in brain smear at autopsy.

TABLE 5.—*Course of* P. gallinaceum *infection in chicks inoculated with blood from a chick of the brain-inoculation (exocrythrocytic) series, and in other chicks infected during subsequent serial passage of these parasites by blood-inoculation: An example of conversion of pattern C to pattern B by changing the inoculum from brain to blood*

Inoculum	Chick no.	Parasitized erythrocytes per 10,000 red blood cells on indicated post-inoculation days								
		3	4	5	6	7	8	9	10	11
Blood from chick of the brain inoculation (exo-erythocytic series)	1	0	0	0	5	30	D–E (Heavy)		....	....
	2	0	0	5	20	D–E (Heavy)		....	....	....
	3	0	0	0	0	10	D–E (Heavy)		....	....
	4	0	0	0	0	10	D–E (Heavy)		....	....
Blood from chick #2, second serial blood inoculation	5	...	....	5	20	130	D–E (Heavy)		....	....
	6	...	....	5	50	D–E (Heavy)		....	....	....
	7	...	....	5	120	1140	D–E (Heavy)		....	....
	8	...	....	0	30	180	D–E (Heavy)		....	....
Blood from chick #7, third serial blood inoculation	9	375	1900	....	D–P†	....	....	....	....	....
	10	445	1755	....	8200	7047	D–E (Moderate)		....	....
	11	665	2300	....	7600	8450	D–E (Light)		....	....
	12	195	910	....	6800	8850	D–E (Light)		....	....
Blood from chick #11, fourth serial blood inoculation	13	...	460	2160	7400	....	....	D–P	....	....
	14	...	414	2190	5420	....	....	5586	....	D–P
	15	...	141	1350	5830	....	....	D–P	....	....
	16	...	5	20	270	....	....	D–P	....	....

* D–E indicates death, exoerythrocytic parasites found at autopsy—degree of infection as indicated.
† D–P indicates death, pigmented erythocytic parasites found at autopsy; no exoerythrocytic forms.

produced higher parasitemias than the first; the third was accompanied by diminution of exoerythrocytic forms during the acute stage; the fourth serial passage by blood-inoculation corresponded entirely with the first phase of pattern B, namely, high parasitemia and no exoerythrocytic forms in chicks dying in the acute erythrocytic phase.

It should be noted that there was a transitional or intermediate pattern during the several passage generations that were required for conversion of pattern C to pattern B: this transitional pattern was marked by features of both C and B, namely high initial exoerythrocytic infection plus early development of parasitemia.

Characteristics of Converted Strain.—In most respects these converted strains resemble the strain passed by blood inoculation. One striking difference is the almost complete absence of gametocytes in the converted strains. There have been three substrains started from the brain emulsion-passed chicks: one from the fourth brain passage, one from the 19th brain passage and one from the 34th brain passage. Because of insufficient data on the last, only the characteristics of the first two converted strains will be discussed. The first sub-strain (E_1) has been carried for 37 passages and the second sub-strain (E_2) has been carried for 20 passages, both by blood inoculation. Parasite counts were made on at least three days on all of the chicks (6) of each passage. Only one gametocyte was seen on the smear of one bird in the 8th passage of the E_1 strain. In contrast to this, although gametocytes were rare in the blood-passed strain, persistent searching reveals their presence fairly frequently.

In spite of the fact that gametocytes were not observed these converted strains retained the ability to infect mosquitoes. In seven lots of mosquitoes fed at intervals on the regular blood inoculation strain (average 23 mosquitoes per lot) there was an average of 20.26 oöcysts per mosquito stomach. There were 12 lots (average 20 mosquitoes per lot) fed on E_1 strain. These mosquitoes had an average of 15.66 oöcysts per mosquito stomach. Although there were only 6 lots (average 28 mosquitoes per lot) fed on E_2 strain they had an average of 56.46 oöcysts per mosquito stomach.

Fresh chicks inoculated with sporozoites from mosquitoes infected on any of the above strains have a parasite picture which follows pattern C. There will be further discussion of this phase later in this paper.

Brain-inoculated Chick Embryos.—An exoerythrocytic type of parasite development, essentially that of pattern C, occurs in chick embryos inoculated with brain emulsion (Haas, Wilcox, Ewing, 1945) either via the yolk sac, or by the chorioallantoic route described by Zuckerman (1946). Using infected embryo brain as the inoculum, this infection has been maintained serially in embryos for more than 90 passages.

Since daily parasite counts are impractical with chick embryos, the pattern of parasite development is not shown in exactly the same manner as with chicks (Haas, Wilcox, Ewing, 1945). Smears of heart blood of 112 embryos dying or killed on the 6th to 12th post-inoculation days showed parasites absent or too rare to count in 80. In the other 32 embryos, there were from 5 to 450 parasitized cells per 10,000 erythrocytes, with a mean of 116. Heavy exoerythrocytic infections were present in all. A constant feature of this type of infection in chick embryos is the failure of the erythrocytic parasites to produce pigment (Haas, Wilcox, Ewing, 1945).

Conversion from Pattern C to Pattern B in Embryos.—When blood from embryos of this series was inoculated into fresh embryos and the resulting infection was subsequently maintained by serial blood-inoculations, there was a gradual conversion of the parasite development from pattern C to pattern B, as was seen with chicks so inoculated. The first blood-inoculated series had heavy exoerythrocytic infections, with small numbers of erythrocytic parasites, all non-pigmented; the second series showed many exoerythrocytic forms, with some pigmented erythrocytic parasites; by the fourth intravenous passage, more than half the erythrocytes contained pigmented parasites, and the number of exoerythrocytic forms had markedly diminished. From the eighth serial blood-inoculation, no more exoerythrocytic forms were seen. The intermediate character of the pattern of parasite development during the transitional stage between pattern C and pattern B is evident.

Sporozoite-inoculated Embryos.—A less clear-cut example of pattern C is seen in embryos infected by sporozoites. In 1940, Shortt, Menon, and Seetharama reported an extremely heavy infection of the brain, and a "moderate" infection of the

TABLE 6.—*Nature of* P. gallinaceum *infection in chick embryos inoculated with sporozoites (mosquito bites) of alternate mosquito-chick passage series, showing parasite development of pattern A in some, pattern C in others, and an intermediate nature in still others*

Embryo number	Day of death	Exoerythrocytic forms in brain smears	Parasitized cells per 10,000 erythrocytes	Parasite development pattern
1	6	Present	0	C
2	6	Present	5	C
3	8	Present	5	C
4	8	Present	2,000	Intermediate
5	9	Present	5	C
6	10	Present	830	C
7	10	Present	120	C
8	10	Present	1,000	Intermediate
9	10	Present	3,000	Intermediate
10	10	Present	7,800	Intermediate
11	10	Present	8,300	Intermediate
12	10	None	9,000	A
13	12	Present	0	C
14	14	Present	5,500	Intermediate
15	14	Present	5,900	Intermediate
16	14	None	7,400	A
17	14	None	7,600	A
18	17	None	9,000	A

erythrocytes, in an embryo which died 11 days after infected salivary glands of an *Aedes aegypti* had been placed on the chorio-allantoic membrane. They reasoned that if the embryo had been killed earlier, exoerythrocytic forms would probably have been demonstrable before the development of pigmented forms.

In embryos inoculated by the bites of mosquitoes from the mosquito-chick passage series (Haas and Ewing, 1945) infections have resembled pattern A in some, and pattern C in others (Table 6). In still others there were concurrent heavy parasitemias and numerous exoerythrocytic forms, constituting an intermediate or transitional pattern.

It appears that in chick embryos, exoerythrocytic forms tend to grow more rapidly and profusely than in chicks, when the source of infection is sporozoites from an alternating mosquito-chick passage series.

If such exoerythrocytic predominance is sufficiently marked, embryos die before erythrocytic parasites become numerous, thus presenting the pattern C development. There is a tendency for erythrocytic forms to become more prominent the longer the embryo survives. If survival is long enough, these may be the only forms seen

at autopsy; the parasite development thus would conform to pattern A. Midway between these extremes, in embryos surviving around 10 days, the transitional pattern may predominate.

Certain Sporozoite-inoculated Chicks.—Chicks inoculated with sporozoites originating in the course of alternating mosquito-chick passage develop pattern A infection. But chicks may also be infected with sporozoites from another source, namely those produced in mosquitoes which have bitten chicks of the blood-inoculated series. Chicks infected by sporozoites from this source develop typical pattern C infections (Haas, Wilcox, Davis, and Ewing, 1946). Neither age of chick (from 2 to 63 days) nor route of inoculation of sporozoites (mosquito bite, salivary glands, or suspension of triturated mosquitoes (Coatney, Cooper, and Trembley, 1945) is a factor (Haas, Wilcox, Davis, and Ewing, 1946).

The contrast between the two patterns of parasite development is evident in Table 7. It may also be shown by inoculating chicks with sporozoites from the two

TABLE 7.—*Course of* P. gallinaceum *infection in chicks inoculated with sporozoites from alternating chick-mosquito series, an example of parasite development pattern A (chicks 1–5), contrasted with course of infection in chicks inoculated with sporozoites from mosquitoes infected on chicks of blood inoculation series, an example of parasite development pattern C (chicks 6–10). Each chick received equivalent of 1 infected mosquito (Coatney, Cooper, and Trembley, 1945)*

Chick no.	Source of sporozoites	Parasitized erythrocytes per 10,000 red blood cells on indicated post-inoculation day								
		7	8	9	10	11	13	14	16	17
1	Mosquito-chick series	0	0	0	5	80	2100	D–P†	....	....
2	" "	0	0	10	20	80	6400	....	5200	D–P
3	" "	0	5	0	10	12	5400	....	6700	D–P
4	" "	0	0	0	30	40	6800	D–P,E	....	....
5	" "	0	5	20	90	630	4100	....	3500	D–P
6	Blood inoculation series	0	0	30	10	D–E*	....	....	....	....
7	" "	0	0	0	D–E	....	....	....	....	....
8	" "	0	0	D–E	....	....	....	....	....	....
9	" "	0	0	5	10	D–E	....	....	....	....
10	" "	0	0	40	D–E	....	....	....	....	....

* D–E indicates death, exoerythrocytic parasites found at autopsy.
† D–P indicates death, pigmented erythocytic parasites found at autopsy.

different sources, and searching for parasites in chicks of the two series which die or are killed at daily intervals after inoculation (Table 8). Such an experiment indicates the early appearance—from the 8th day onward—of exoerythrocytic forms in the brains of chicks whose infection was derived from the blood-inoculated parasites, as contrasted with the failure to find such forms in chicks of the alternating chick-mosquito group.

The regularity with which each type of infection develops according to its characteristic pattern was noted in our original report (Haas, Wilcox, Davis, and Ewing, (1946), where exoerythrocytic type of infection was present in 46 out of 58 chicks inoculated with sporozoites derived from blood-inoculated parasites, but in only 3 out of 244 chicks of the alternating mosquito-chick series. In our subsequent experience, the constant nature of these patterns has been continuously apparent.

It has been noted above that pattern C infections resulting from brain-inocula-

TABLE 8.—*Course of* P. gallinaceum *infection in chicks inoculated with sporozoites from alternating mosquito-chick series (pattern A) contrasted with that in chicks inoculated with sporozoites derived from blood-inoculation series (pattern C). Three chicks examined daily by blood and brain smears. Chicks examined on 3rd to 12th day received equivalent of 1 whole infected mosquito intramuscularly (Coatney, Cooper, and Trembley, 1945); others received 3 mosquitoes*

Chicks examined daily	Source of sporozoites	Parasitized cells per 10,000 erythrocytes expressed as numerator, and type of parasites found in brain smear expressed as denominator, on indicated post-inoculation days													
		3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	Mosquito-chick series	0/0	0/0	0/0	0/0	0/0	0/0	1200/P*	0/0	5/P	10/P	3200/P	4500/P	2400/P	30/P
2	"	0/0	0/0	0/0	0/0	0/0	0/0	10/0	5/0	5/P	20/P	7500/P	3800/P	1200/P	80/P
3	"	0/0	0/0	0/0	0/0	0/0	0/0	10/0	30/0	10/0	....	2900/P	5200/P	5700/P	....
4	Blood-inoculation series	0/0	0/0	0/0	0/0	0/0	0/E†	5/E	0/E	0/E	No survivors beyond 11th day				
5	"	0/0	0/0	0/0	0/0	0/0	0/0	30/E	0/E	0/E	"	"	"	"	"
6	"	0/0	0/0	0/0	0/0	0/0	0/E	0/0	0/E	0/E	"	"	"	"	"

* P indicates pigmented erythrocytic forms found in brain smear—no exoerythrocytic forms.
† E indicates exoerythrocytic forms found in brain smears.

tions, both in chicks and in chick embryos, may be converted to pattern B by several serial passages with blood instead of brain inocula. Similarly, a pattern C infection produced by sporozoites derived from the blood-inoculated chicks was converted to pattern A by several serial passages through mosquitoes and chicks in regular alternation. To accomplish this, it was necessary to inoculate many chicks initially, in order to get one or two which lived long enough to produce gametocytes, but once this was done, conversion from pattern C to pattern A took place by the third serial passage (Haas, Wilcox, Davis, and Ewing, 1946).

Sporozoites derived from the blood-inoculation strain maintained in chick embryos produced in chicks the same pattern C infection as those derived from the blood-inoculation strain maintained in chicks.

The occurrence of exoerythrocytic forms in the brains (and other organs) of sporozoite-inoculated chicks at about the time of patency of the blood infection has been remarked by James and Tate (1938), by James (1939), by Shortt, Menon, and Seetharama (1940), and by Coatney, Cooper, and Miles (1945). James and Tate (1938) stated that exoerythrocytic forms were not found in mosquito-bitten chicks prior to the appearance of erythrocytic forms. On the other hand, Shortt, Menon, and Seetharama (1940) found these forms in the brains of chicks killed on the 6th and 7th post-inoculation days, when blood infection was either absent or very light. Coatney, Cooper, and Miles (1945) found exoerythrocytic forms in brains on the 5th and 6th days, before patent blood infection had developed, though such instances were rare. Later, Coatney, Cooper, and Trembley (1945) described what appears to correspond with what we now designate pattern C infection: "Brain smears of birds dying of sporozoite-induced infection show most of the capillaries packed with exoerythrocytic parasites, while the erythrocytic infection is often not at a level that would produce detectable symptoms, much less death."

Our experience supports all these views: we have seen chicks infected by sporozoites in which no exoerythrocytic forms could be demonstrated (by us) in the brains (Tables 1 and 2), and we have seen chicks infected by sporozoites in which exoerythrocytic forms were numerous before and at the beginning of patency of the erythrocytic infection (Tables 7 and 8). In our experience, the determining factor is the origin of the sporozoites: those derived from alternating mosquito-chick passage produce an infection in which erythrocytic parasites predominate (pattern A), while those derived from a blood-inoculation series produce an infection characterized by predominance of exoerythrocytic forms (pattern C).

The point to remember is that the results described here have been obtained with parasites which have been strictly maintained by the two different methods of passage, i.e., alternating mosquito-chick passage and serial blood-transfers, for several years. No crossing over from one strain to another has occurred. In such circumstances, the difference between the patterns of infection produced by sporozoites from the two strains is clear cut. Where no such distinctly separate strains are maintained, transitions from one pattern to another would be expected, in view of our experience in converting one clear cut pattern to another entirely different pattern by altering the method of transfer. That intermediate patterns occur under such circumstances is clear from what has already been said. It is not surprising that observations on the development of exoerythrocytic forms have differed somewhat;

the explanation, in view of our experience, lies in the source from which the sporozoites in any given case were derived.

It is emphasized that pattern A, in which the exoerythrocytic forms play an unobtrusive though essential role, is the "natural" pattern of development. As alterations in the life cycle, e.g., blood-inoculation, are introduced, departures from this pattern appear. Pattern A is the only type of development that could be consistent with the natural history of *P. gallinaceum,* which requires that a number of chicks survive long enough to develop gametocytes. Where profuse growth of exoerythrocytic forms kills the warm-blooded host before parasitemia develops (pattern C), no gametocytes are found, and cyclical transmission of the parasite, as in nature, is impossible. (We have tried to infect mosquitoes on chicks with pattern C infection, but have been unable to do so.)

Production of Pattern C by Use of Quinine.—Predominance of exoerythrocytic forms in chicks whose erythrocytic parasitemia had been suppressed with quinine was remarked by James and Tate (1938). In 1941, Adler and Tchernomoretz described a type of infection which corresponds to pattern C. They obtained this pattern of parasite development by suppressing erythrocytic forms with quinine, and were able to maintain it in serial passage. Their inoculum was an emulsion of brain, liver, spleen, and kidney, and when quinine was omitted, or reduced in quantity, pigmented parasites became prevalent. Presumably the large blood content of the liver, spleen, and kidney included in their inoculum permitted early development of the erythrocytic infection, just as occurs with blood-inoculation. In order to produce an exoerythrocytic predominance under these conditions, it was necessary to suppress the erythrocytic parasites with quinine, thereby allowing the exoerythrocytic forms, which are particularly numerous in the brain—an organ included in their inoculum—to assume predominance.

SUMMARY

Three patterns of parasite development in *P. gallinaceum* infection of chicks and chick embryos have been described:

Pattern A.—Seen in chicks of an alternating chick-mosquito series. Exoerythrocytic forms occur before the parasitemia, and play an essential but unobtrusive role. This pattern may be regarded as the one which occurs in nature.

Pattern B.—Seen in blood-inoculated chicks. Erythrocytic forms occur first; exoerythrocytic forms appear later in chicks surviving the initial parasitemia. This pattern represents a response to an alteration of the life cycle of the parasite.

Pattern C.—Heavy exoerythrocytic infection early, causing death of the chick before erythrocytic parasite development gets under way. Occurs:

(a) In chicks inoculated with emulsions of brains containing exoerythrocytic forms. Transmissible in series.

(b) In embryos similarly inoculated. Also transmissible serially. Accompanied by non-pigmented erythrocytic forms.

(c) In some embryos infected by mosquitoes of the alternating mosquito-chick series.

(d) In chicks infected by sporozoites derived from parasites of a blood-inoculated strain.

(e) In chicks in which erythrocytic forms are suppressed by quinine.

These patterns of parasite development are remarkably regular as long as the particular method of maintaining the parasite is followed in each instance. When a change in method of transferring parasites from one host to another is instituted, it results in a change in the pattern of parasite development, the pattern characteristic of the former method of maintenance giving way to that typical of the latter. This change is gradual, requiring from 3 to 8 passage generations, and is marked by patterns of parasite development of an intermediate nature during the period of transition.

If the cyclical existence of *P. gallinaceum* which nature provides, i.e., alternating mosquito-chick transmission, were the only one possible, the pattern of exoerythrocytic schizogony would have appeared simple and logical from the time of its first recognition. Because man has devised methods of altering this cyclical existence, patterns of development occur which were doubtless never contemplated in the natural history of this parasite.

James (1939) expressed the idea that the exoerythrocytic forms were an indication of an alternate method of schizogony, which under certain circumstances could take the place of erythrocytic parasite schizogony. In nature, this exoerythrocytic schizogony may be regarded as taking the place of erythrocytic parasite schizogony before patency, and probably also during periods of latency. Under the abnormal circumstances responsible for patterns B and C, this alternate method of schizogony represents the response of the parasites to unnatural conditions.

The regularity with which these deviations from the natural pattern occur under artificial conditions indicates the marked effect which is produced upon the development of the parasite by alterations in the life cycle. At the same time, the relative rapidity with which one pattern may be converted to another by a simple alteration in the life cycle, indicates that the changes produced by such alterations are not of a profound or lasting nature.

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EXPERIMENTAL TRANSMISSION OF THE "3M" STRAIN OF *PLASMODIUM CATHEMERIUM* TO THE DUCK AND ITS CHEMOTHERAPEUTIC SUITABILITY FOR ROUTINE ANTIMALARIAL SCREENING[1]

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MATERIALS AND METHODS

Laboratory hosts consisted of adult female canaries (*Serinus canarius*) weighing about 15 grams and white Pekin ducks obtained when one day old, and raised until they weighed about 100 grams. Chicks used were 3- to 5-day-old white Leghorns weighing 40 to 50 grams.

A rotary feed mixing machine was used to mix the drug diets (Litchfield et al, 1939) employed in the chemotherapeutic studies. Thorough mixing was achieved in 30 minutes. Separate containers for running water and food were designed to accommodate the various groups of 3 ducks each for the chemotherapeutic studies. Thus, it was impossible for the running water and feed to become mixed with that of another group of ducks on tests. Ten groups were put on test at one time. The duck pens were placed on metal racks with one-half inch hardware cloth floors, thus allowing the fecal material to fall on a metal apron. The fecal material then passed down an outlet to the sewer by the action of the continuously running water, which was the overflow from the drinking troughs.

For the study of periodicity, synchronicity, and course of infection, the ducks were kept on a 12-hour light and a 12-hour dark cycle in a regular type brooder pen.

The Lumetron colorimeter, calibrated against an Evelyn hemoglobinometer, was used to determine the hemoglobin as oxyhemoglobin (Coffin, 1946).

The method of counting the number of parasites in relation to red blood cells is that calculated by Hartman (1927) and Gingrich (1932). A Howard disc was used to facilitate counting.

Canary no. 80 was bled intracardially and the parasites per 10,000 red cells was determined. One-half ml. of the blood containing 40×10^6 parasites was quickly frozen in small brown glass tubes with very thin walls by immersing them in a mixture of dry ice and acetone. These were then stored in a deep freeze unit at $-70°$ F. The remainder of the blood containing 40×10^6 parasites was given to two one-day-old ducks via the tarsometatarsal vein, each duck receiving 20×10^6 parasites. Initial experiments carried out indicated that the "3M" strain of *Plasmodium cathemerium* would be readily adapted to the duck.

Once the strain was well established in the duck, initial experiments were carried out using 1×10^6 parasites per gram of body weight. This produced a

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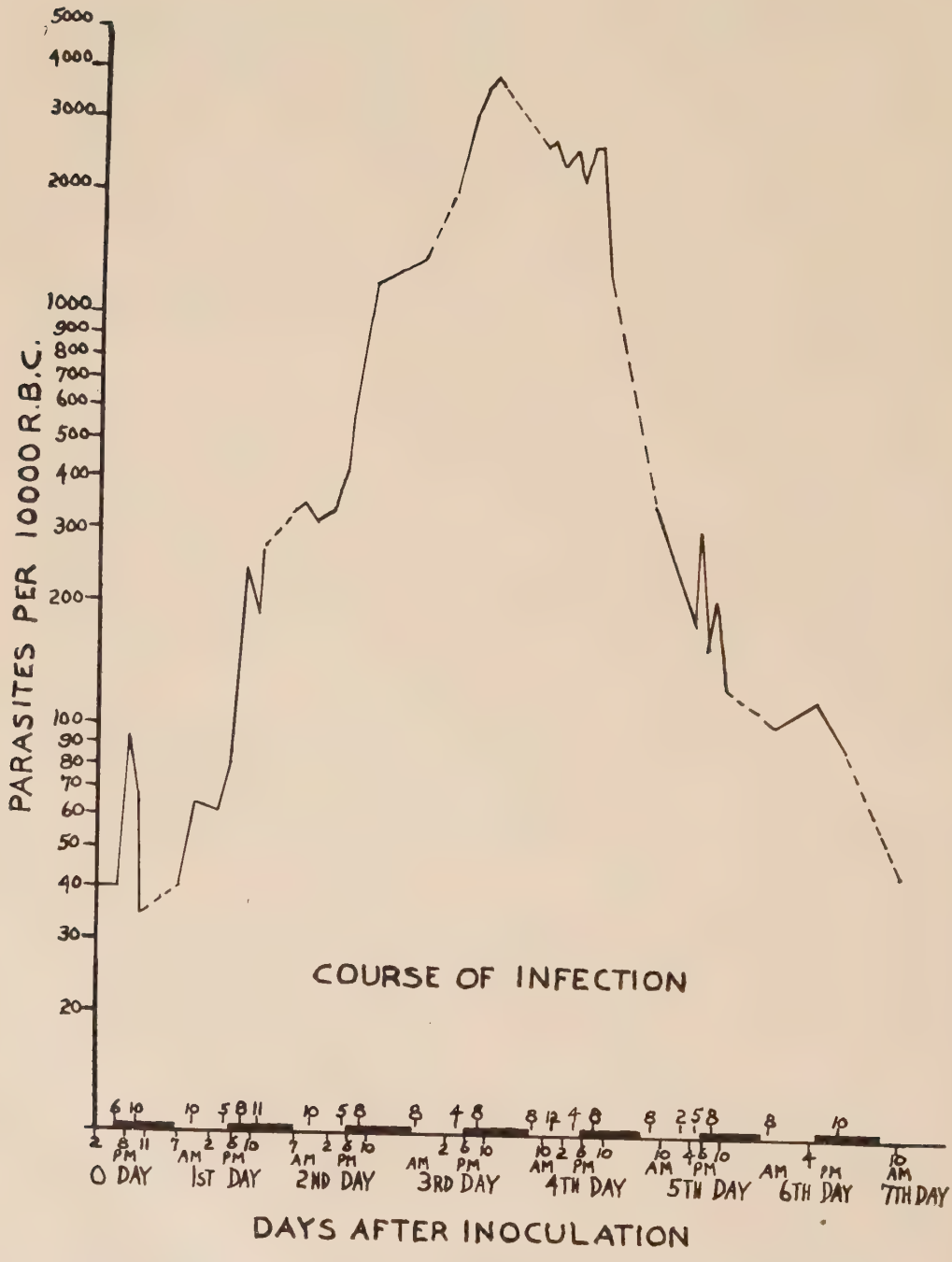


GRAPH 1. The mean course of infection throughout the patent period with the "3M" strain of *Plasmodium cathemerium* in ducks.

sufficiently high parasitemia for a study of periodicity, but was considered low for chemotherapeutic studies. This dosage produced a parasitemia with a range of 3,800 to 6,500 parasites per 10,000 red blood cells.

EXPERIMENTAL DATA

Peak of Infection

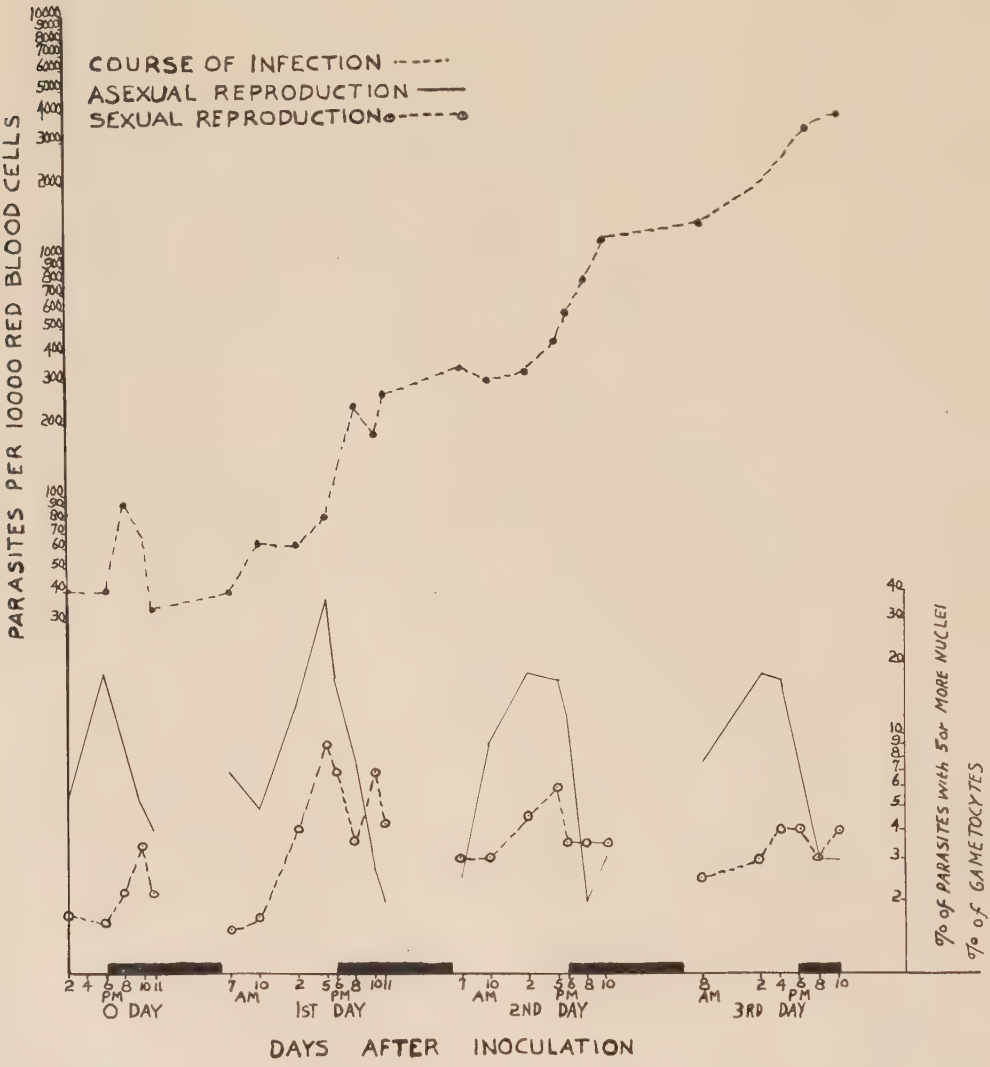
Slides were made at two-hour intervals during the day in order that the periodicity could be determined at the same time. Four ducks were used in the combined peak of infection and periodicity study. The ducks were injected with 1×10^6 parasites per gram of body weight at 11:00 AM and smears were taken at 2:00 PM the same day and every two hours until 10:00 PM, and in some instances 11:00 PM. Initial counts showed that all of the birds had received desirable amounts of parasites and that the parasitemia of the various birds were coming up with a close correlation. Due to individual responses to malarial infections and biological variations in phagocytosis and immunity, it was decided that the geometric mean gave a better overall picture because it is a calculated value and is dependent upon the size of all the values.

Graph 1 represents the mean course of the infection in 4 ducks. The peak parasitemia was reached the third day at 10:00 PM following the injection of 1×10^6 parasites per gram of body weight in all of the ducks studied. The donor blood used in the experiment was from ducks which were on a 12-hour light and a 12-hour dark schedule (Boyd, 1929). These data correspond with the results Wolfson (1943) obtained for the "3T" strain of *Plasmodium cathemerium* in the duck. However, Wolfson (1943) states ". . . that further rapid passage of this strain through the duck is likely to increase the degree of parasitemia, to shift the peak to a later time in the infection, and to cause more frequent deaths of the host." It has been found by the author that if the number of parasites injected remains constant, there is no increase in the degree of virulence of the "3M" strain of *Plasmodium cathemerium* in over 300 transfers. The degree of virulence appears to be, to a certain extent, proportional to the number of parasites injected intravenously when the hosts are in good condition and the parasites are obtained from the donor before the peak of the parasitemia is reached.

Periodicity and Synchronicity

The periodicity study was carried out in the following manner: smears were made at two-hour intervals, and in some instances at one-hour intervals, and stained with Giemsa's solution. During the period between 4:00 PM and 8:00 PM, smears were usually made every hour. From a random sample of 100 parasites from each blood smear the percentage of each of the following groups was determined: (1) stages containing 5 or more nuclei which were designated as old schizonts, (2) microgametocytes, and (3) macrogametocytes. There was a great deal of similarity in the 4 birds studied and Graph 2 depicts the results of the periodicity study. The periodicity was essentially 24 hours in length. On the day of transfusion, the maximum number of schizonts with 5 or more nuclei occurred at 6:00 PM; the day after inoculation, or the first day of the infection, the maximum number of these schizonts occurred at 5:00 PM. The second day of the infection the peak becomes flattened somewhat and the maximum number of

old schizonts occurred at 2:00 PM, and the third day was like the second day. Segmentation occurring earlier in the afternoon in the peripheral blood was noted by Wolfson (1938), by Redmond for the "3H" strain, and by Coatney for the "3M" strain (unpublished), and by Porter (unpublished) for the "3M" strain.



GRAPH 2. Asexual and sexual periodicity of the "3M" strain of *Plasmodium cathemerium* in the duck. Segmentation occurring earlier in the afternoon was noted on the second and third days of the infection.

Those observations of earlier segmentation for the various strains mentioned were on infections in the canary.

Periodicity of the gametocytes was also studied and recorded on Graph 2. The male and female gametocytes appeared in the peripheral circulation simultaneously with the asexual forms, which might be expected with the use of a large inoculum. The gametocytes were observed to show a periodicity quite similar to that of the asexual forms. However, the maximum number of game-

tocytes appeared at a later hour than the old schizonts. This would tend to indicate that the schizonts and gametocytes reached maturity at nearly the same time. These observations were in agreement with those of Shah (1934). Criteria for distinguishing pregametocytes, microgametocytes, and macrogametocytes were based on the cytological characteristics offered by Gambrell (1937).

Synchronicity appeared to be quite high despite the fact that segmentation occurred at an earlier hour on the third and fourth days of the infection. The synchronicity was much sharper on the first and second day of the infection with the synchronicity appearing to be low near the peak of the infection. Following the peak of the infection, synchronicity was nearly broken up completely, and multiple parasites were found in the red blood cells, the polychromatophilic erythroblasts (Hegner and Hewitt, 1938; Hegner and Eskridge, 1938a; Hewitt, 1939) more than at any other time.

Number of Merozoites Produced per Schizont

Thirty segmenters were counted in each of the 4 slides made 77 hours after the ducks were injected intravenously with the malarial blood. The method of choosing the segmenters was by random sampling. Only those segmenters were counted which showed complete division of the cytoplasm and no evidence of further division. The merozoite counts were not made during the first and second days of the infection for several reasons among which were: the first day counts may be unduly influenced by such factors as nutrition, etc., imposed upon it by the donor bird, and immunity near the peak of the infection in the donor bird, all of which might be reflected in the recipient at such an early date. Table 1 shows the results obtained.

TABLE 1

Bird no.	Am	Range	Std. Dev.	Std. Er.	Co. of Var.	Std. Er. Co. of Var.
16C	11.00	8–14	1.79	± 0.32	16.2%	± 2.1
17C	10.83	6–16	2.73	± 0.49	25.0%	± 3.4
18C	13.17	8–24	3.95	± 0.72	29.0%	± 4.0
19C	12.37	6–18	3.12	± 0.56	25.0%	± 3.4

The merozoite mean of the 4 ducks ranged from 10.83 to 13.17, and the actual numbers ranged from 6 to 24. The average number of merozoites for the 4 birds was 11.84 ± 0.29 with a standard deviation of 3.13.

The destruction rate of the "H" strain of *Plasmodium cathemerium* in the canary has been determined by Boyd (1939). He found that at the peak of infection the destruction rate was around 90%. The destruction rate was determined for the "3M" strain of *Plasmodium cathemerium* in the duck in the following manner. The number of parasites per 10,000 red blood cells was determined from smears made at 2:00 PM on the third day of the infection. Merozoite counts were made at 4:00 PM on the same day, and the two multiplied together. This would give the population per 10,000 red blood cells which should theoretically exist in the next 24-hour period. The number of parasites determined for the 4 ducks was 1,400, 2,305, 3,082, and 1,449, respectively, for ducks 16C, 17C, 18C, and 19C. The number of merozoites has been reported for the ducks. Hence, the per cent of destruction was as follows: duck 16C, 90%; 17C, 90.1%; 18C, 96.7%; and 19C, 59%. This high ratio of parasite destruction indicated the extent of acquired immunity at the crisis of the infection.

Exoerythrocytic Stages

Corradetti (1940) drew the conclusion that the exoerythrocytic cycle was a test of the degree of adaptation reached by each species in its relation to its respective host. Hewitt (1940) stated that, "if an exoerythrocytic schizogonic cycle does form a part of the life cycle of some strains of avian plasmodia, the factors which govern the appearance of such a cycle are biologically unstable." Wolfson (1940) observed that exoerythrocytic stages were prevented in the canary by passage through ducks, and no exoerythrocytic stages were found in the duck associated with the "3T" strain. Porter (1942) found a similarity between strains "3M," "3A," and "3T" of *Plasmodium cathemerium,* as all showed exoerythrocytic schizogony during blood passage after recent passage through mosquitoes, and all were markedly virulent for canaries.

In the present search for exoerythrocytic stages, 24 birds have been examined. The organs routinely examined were the brain, heart, lungs, liver, spleen, bone marrow, and occasionally the muscle. No exoerythrocytic stages were found. However, many parasites were seen outside of the red blood cells, and in the brain especially. The parasites seen were usually immature schizonts and gametocytes. These were undoubtedly the result of rupture of the red blood cells, because they contained malarial pigment.

Induced "3M" strain of Plasmodium cathemerium in Chicks

Six chicks were used in the experiment, ranging from 40 to 50 grams. The dosage of parasites ranged from 1×10^6 parasites per gram of body weight to 450×10^6 parasites per gram of body weight. In all cases the results were the same. Parasite counts remained nearly constant in all of the chicks for 3 days; thereafter, there was a marked decline until their disappearance. This evidence of the resistance of chicks to the "3M" strain coincided with the results of Manwell (1933), and Hegner and West (1941) using various other strains of *Plasmodium cathemerium.*

Chemotherapeutic Experiments

Drugs on test were made up in the feed (Litchfield et al, 1939). As ducks ate only during periods of light, a 4-hour light and dark schedule was followed. The time of initiation of treatment was 24 hours before infection. The duration of treatment with the "3M" strain of *Plasmodium cathemerium* was 5 days. The size of the inoculum was 1.5×10^6 parasites per gram of duck, which was given intravenously. The mean weight of the 30 ducks used on the test was 119 grams with a range of 84 to 142 grams. Hemoglobins were determined by the method described. The mean hemoglobin value for the 30 ducks was 9.7 gm% with a range of 8.9 to 11.87 gm%. Six ducks were used as controls (groups 1 and 2), and the 24 remaining ducks were placed 3 to a pen and put on the drug diets shown in Table 2.

Hemoglobin determinations were made before inoculation and on the third, fourth, fifth, sixth, seventh, and twelfth day of the infection at 10:00 AM.

Thin blood smears were made at 8:00 AM on the first, third, fourth, and fifth days after injection of 1.5×10^6 parasites per gram of body weight.

Groups 1 and 2 (controls) reached a parasitemia peak on the fourth day of

the infection with a count of 3,352 and 3,705 parasites per 10,000 erythrocytes. The geometric mean of the two groups was 3,524.

Groups 3, 4, and 5 (quinines) reached a peak on the third day. Group 3 ducks on 0.0125% drug diet (minimum effective dose) had a parasitemia of 1,695 parasites per 10,000 red blood cells. Group 4 receiving 0.025% quinine had a peak of 1,409 parasites, while group 5 on 0.05% quinine had a peak of 439 parasites per 10,000 red blood cells.

Ducks on group 6, PAM 23, 0.0125%, reached a peak on the fourth day, as did the controls, with a peak parasite count of 4,031. This dosage of drug was apparently a subeffective level.

Group 7 on sulfadiazine reached a peak on the fourth day with a count of 2,039 parasites, although the peak tended to be flattened out. Sulfadiazine was put on test as a check to determine if this strain of parasite was sulfa-susceptible.

Group 8 on PAM 2–4–2 received 0.0008% drug in the diet. This group of ducks reached a peak parasitemia on the fourth day of infection with 2,134 parasites. Marked morphological changes were noted in the parasites, many appearing "punched out" and dark in appearance. The number of merozoites

TABLE 2

Bird group no.	Drug	Drug diet %
1.	Control	0.0
2.	Control	0.0
3.	Quinine	0.0125
4.	Quinine	0.025
5.	Quinine	0.05
6.	PAM 23*	0.0125
7.	Sulfadiazine	0.8
8.	PAM 2-4-2*	0.0008
9.	PAM 3-4-b*	0.00078
10.	Atebrin	0.0014

* Confidential Parke, Davis, and Co. antimalarials.

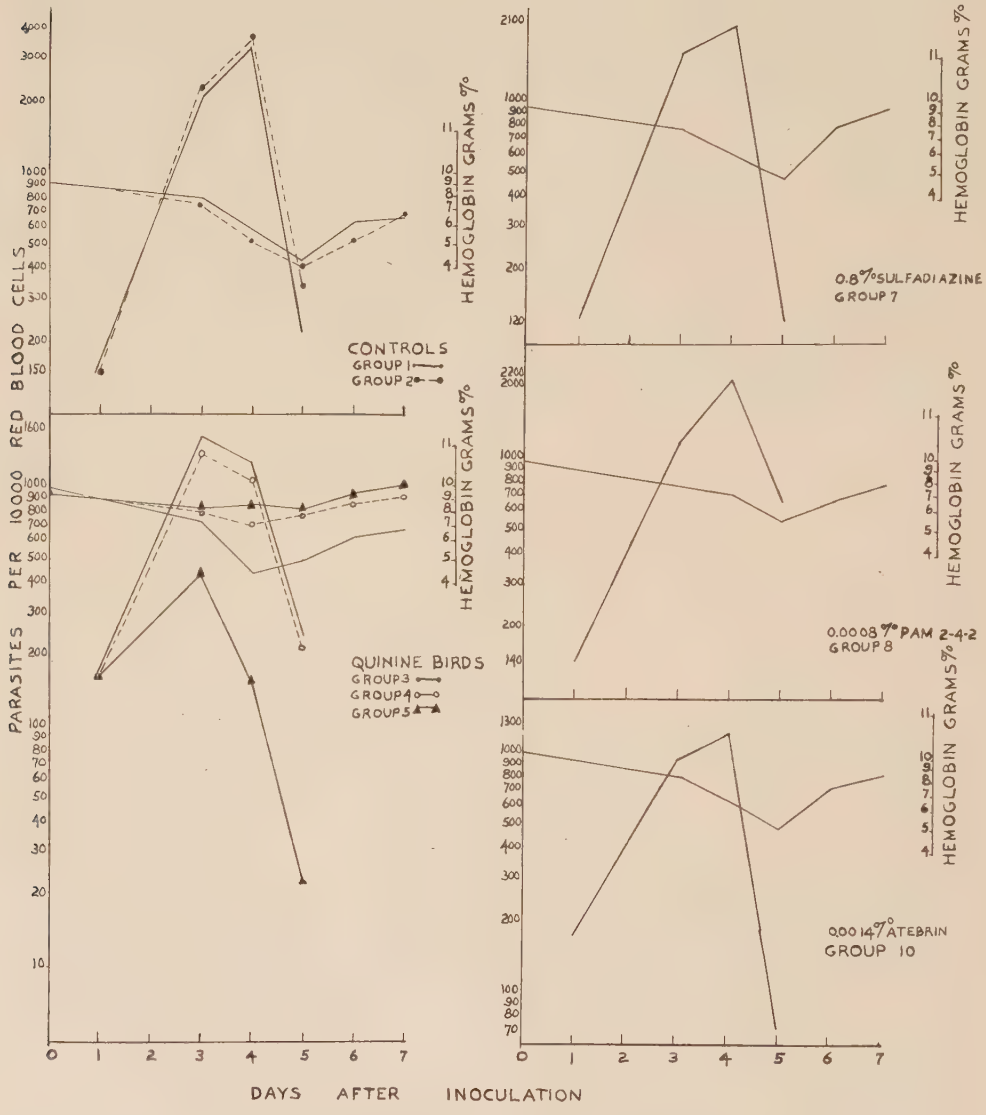
were noted to be fewer in number and there appeared to be good gametocidal action. The dosage of this drug was the minimum effective level. The counts were suppressed 50% that of the controls and the quinine equivalent was 15.

Group 9 on PAM 3–4–b, 0.00078% drug, never reached a peak. The amount of drug employed completely suppressed the infection, preventing a peak of any kind. From this preliminary test, the drug had a Q 64^{+}. Gametocidal and schizonticidal action was most apparent. Gametocytes and schizonts appeared as dark shrunken spots in the red blood cells. Morphological characteristics were obliterated. The maximum number of merozoites discernible was 4 or 5, and this number could be considered as only approximately accurate as the schizonts were so dark.

Ducks on group 10 receiving 0.0014% atebrin reached a peak on the fourth day with 1,240 parasites per 10,000 red blood cells. Thus a Q 17 is in the range reported by other workers. This drug was put on test as merely a drug control, the same as sulfadiazine, to determine if there were any manifestations of hypersensitiveness or idiosyncrasies by the parasite to those drugs already reported on.

Parasite counts of ducks on drug diets PAM 23, sulfadiazine, PAM 2–4–2, atebrin and the controls all reached a peak on the fourth day of the infection and all showed the same type of parasitemia curve. That is, the parasitemia progressed to the third day with a straight line approach and then flattened out

before the peak and dropped rapidly after the peak. In contrast, the quinine curves revealed a rapid rise to a peak and then flattened out on the other side of the peak. The characteristics of the two types of curves may or may not be significant.



GRAPH 3. Parasitemia and anemia curves of the untreated controls and treated ducks on various minimum effective levels of drugs during the chemotherapy studies.

Also worthy of mention is the variation in the morphology of the avian malarial parasites under drug therapy. The avian malarial parasites in ducks receiving quinine exhibited a definite and consistent chemotherapeutic damage. That is, they appeared "punched out" and somewhat faded. In contrast, the parasites in ducks receiving PAM 3-4-b showed a different type of damage. Their morphology was altered from the normal in that they were extremely

dark and some were black, and appeared to be what I shall term "chemotherapeutically burned." It is therefore suggested that the morphological alterations of the avian malarial parasite under drug therapy may be a significant factor from a chemical and biological viewpoint. As desirable types of drug action, i.e., detrimental to a particular system of the parasite which would be irreversible, etc., drug resistance and reproductive ability, both sexual and asexual, could be noted.

Ben Harel (1923), Young (1937), Terzian (1941), and Hewitt (1942) demonstrated a fall in the red cell number accompanied by a diminution in hemoglobin. Hewitt, Richardson, and Seager (1942) conducted experiments involving a considerable number of ducks and concluded that "the fall in hemoglobin following different doses of parasites follows the parasite curve very closely . . . and serves as a supplementary method for estimating the degree of parasitemia." Then, too, they found that the sharp drop in hemoglobin on the day after the peak (*P. lophurae*) was evidence *per se* to denote the day on which the highest number of parasites was reached, even though parasite counts were not made.

Anemia and chemotherapeutic experiments run concurrently confirmed the

TABLE 3

	Before inoculation	Peak of anemia	Loss of hemoglobin
	gm %	*gm %*	*per cent*
Group 1	9.34	4.40	52.9
Group 2	9.45	4.20	55.6
Group 3	9.87	4.51	54.4
Group 4	9.56	7.15	25.3
Group 5	9.45	8.02	15.2
Group 7	9.45	4.90	49.2
Group 8	9.56	5.60	41.5
Group 10	10.11	5.04	50.2

Groups 6 and 9 were omitted purposely from Graph 3 and Table 3 because only minimum effective levels of drugs were compared with quinine. Group 6 was a subeffective level, while group 9 was above the minimum effective level.

results of Hewitt, Richardson, and Seager, using the "3M" strain of *Plasmodium cathemerium*. The anemia and parasitemia curves of the controls and ducks on test revealed a very close inverse correlation. The lowest hemoglobin values were on the fifth day of the infection, which was the day following the peak of the infection in all cases except the birds on quinine diets. These quinine birds (groups 3, 4, and 5) reached a peak parasitemia on the third day of the infection and the greatest anemia occurred on the following day (4th day). In contrast *Plasmodium lophurae* infections in the duck are asynchronous, and the peak of the infection, using 1×10^6 parasites per gram of body weight, may be on the fifth or sixth day, while *Plasmodium cathemerium* was constant in that the peak was always reached in the fourth day in the untreated controls.

Results of the chemotherapeutic and anemia study are presented in Graph 3. Table 3 represents the hemoglobin values before inoculation, peak of the anemia, and the percentage of the hemoglobin lost.

By the seventh day many of the ducks had hemoglobin values which were near their preinoculation levels, and by the twelfth day all of the ducks were at their preinoculation levels, and some were above those levels.

SUMMARY AND CONCLUSIONS

The "3M" strain of *Plasmodium cathemerium* has been adapted to the duck and some of its outstanding characteristics are presented. This strain is quite

virulent for the duck as the infection produced a marked degree of anemia in untreated birds. However, once the peak of the infection is reached, the parasites disappear from the blood rapidly.

The parasitemia was standardized so that about 40% of the red blood cells are parasitized. For the study of periodicity 1×10^6 parasites per gram of body weight produced a satisfactory infection which was easily followed and observed. However, for routine antimalarial screening a dosage of 1.5×10^6 parasites per gram of body weight was more desirable.

Observations of over 300 transfers and studies indicate that there is no lethal agent (Dearborn, 1946) associated with the "3M" strain of *Plasmodium cathemerium*. Untreated controls and ducks surviving initial attacks were put into finishing batteries and none has died, except by euthanasia, when crowded conditions deemed it necessary. Therefore, the "3M" strain could be substituted for the "3T" strain in the duck if the lethal agent associated with the "3T" strain became troublesome during relapse studies.

Asexual periodicity of the "3M" strain of *Plasmodium cathemerium* was found to be essentially 24 hours in length. Segmentation occurred earlier in the afternoon on the second and third days of the infection. Gametocyte periodicity followed that of the asexual cycle very closely, but the maximum number of gametocytes produced appeared to be about an hour later. Synchronicity was very high during the period before the peak of infection was reached. Near the peak and thereafter, the synchronicity was broken up to a great extent. Also, multiple infected immature red cells were more numerous than at any other time.

The number of merozoites was 11.84 ± 0.29, 77 hours after inoculation.

Of considerable interest was the fact that if a line were drawn through the points at 2:00 PM on the first, second, and third days of the course of infection graph a straight line would result. Thus, the course of the infection followed the expontential growth law, since the parasite counts were plotted on semilog graph paper. The use of semilog paper obviates the necessity of looking up the log of Y. The horizontal rulings on the paper are drawn to such a scale that the plotting of the original data results in a straight line, if the data follow the exponential growth law (Snedecor, 1946).

Hemoglobins were determined on the uninfected ducks before inoculation and found to average 9.7 gm% for ducks with a mean weight of 119 grams. The oxyhemoglobin method of determining hemoglobins was used in all instances.

Chicks were unsuitable hosts for the "3M" strain of *Plasmodium cathemerium* since the infection was transient in nature.

This strain is not sulfa-susceptible. Atebrin and sulfadiazine have a Q equivalent the same as that reported for the "3T" strain. Therefore, it is believed that the "3M" strain is a suitable malarial parasite in the duck for routine avian antimalarial screening using the method and dosage outlined. Worthy of mention are the altered morphological characteristics of the avian malarial parasites under various drug diets. This fact may be highly significant from a chemical as well as a biological viewpoint in that certain types of damage to the parasite may be indicative of a desirable type of irreversible action upon a particular system of the parasite.

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RICTULARIA DIPODOMIS N. SP. (NEMATODA: THELAZIIDAE) FROM THE KANGAROO RAT *DIPODOMYS* SP.

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Among a series of *Rictularia* of various species from the U. S. National Museum Helminthological Collection loaned to the writer by Dr. E. W. Price was a bottle of fragments of females, apparently undescribed, from a kangaroo rat. Although there were no complete specimens of this nematode, longer pieces included the vulva and the anterior or posterior end, making it possible to compute an approximate length, and to count the combs and spines. The material had been collected from *Dipodomys* sp. at the Climatic Station, Santa Rita Mts., 3,900 ft., 35 mi. S. Tucson, Pima County, Arizona, by C. W. Quaintance, August 5, 1931. Dr. D. F. Hoffmeister, Assistant Curator, Museum of Natural History, University of Illinois, examined the measurements included by the collector ("252-142-36") and reported that ". . . this kangaroo rat, for which the specimen apparently is no longer extant . . . is most likely *Dipodomys merriami.* The small hind foot (36 mm) is particularly suggestive of the Merriam kangaroo rat, *D. merriami.*"

The following description is based on glycerin cleared material:

Rictularia dipodomis n. sp.
(Figures 1 to 5)

Female: Length about 21 mm; maximum width anterior to vulva 0.12 mm, posterior to vulva 0.30 mm. Rather marked constriction present just posterior to stoma in anterior cervical region. Behind this constriction body diameter gradually increases (Fig. 1). Cephalic zone 0.066 mm, and constriction 0.053 mm in diameter. Thick-walled buccal capsule 0.026 mm long, with external diameter of 0.040 mm; anterior edge surrounded by circlet of about 18 denticles (Fig. 3). General arrangement of cephalic papillae as in other members of genus. Lateral papillae of inner circle slightly ventral to level of amphids. Oral opening almost directly anterior. Esophagus 3.26 mm long and terminating in front of vulva which lies 5.41 mm from anterior end. Cervical papillae behind 7th comb pair, 0.390 mm from anterior end. Excretory pore 0.330 mm, nerve ring 0.190 mm, from anterior end. Transition from combs to spines very gradual (Fig. 4); forty pairs combs present, all anterior to vulva, and 33–34 pairs of spines, all posterior to vulva. Maximum length combs 0.100 mm, spines 0.100 mm. Total number cuticular processes 73–74 pairs. Last spine 2.15 mm from tail tip. Combs (Fig. 2) with a more heavily striated appearance than other North American species known from rodents. Tail 0.132 mm long (Fig. 5). Eggs 0.046 × 0.030 mm; embryonated.

Host: Dipodomys sp., probably *merriami.*

Location: Intestine.

Locality: Santa Rita Mts., 35 mi. S. Tucson, Arizona.

Type specimens: U. S. National Museum Helminthological Collection No. 41366.

Dollfus and Desportes (1945) have listed comb and spine numbers of 34 species of *Rictularia* in tabular form on pages 10 and 11 of their paper. The comb counts of several of the species tabulated seem to approximate the count for *R. dipodomis.* However, the original, or subsequent, species descriptions show that in *R. harrisi* Baylis 1934, *R. aethechini* Le Roux 1930, *R. plagiostoma* (Wedl, 1861) (cf. Jaegerskioeld, 1909) and *R. caucasica* Schulz 1927 the oral opening is dorsally inclined. This common characteristic eliminates the likelihood that any of these four species

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is con-specific with *R. dipodomis*. Baylis (1934) did not picture the *Rictularia* sp. which he briefly described from *Aethosciurus byatti*. However, his statement that it closely resembled *R. harrisi* and *R. taterilli* Baylis 1928 would indicate that it agrees with them as regards the inclined oral opening.

The following species were not included in the tabulation of Dollfus and Desportes: *R. nycticebi* (Mönnig 1920), *R. vulpis* Galli-Valerio 1932, *R. muris* Galli-Valerio 1932, *R. elegans* Travassos 1928, *R. oligopectinea* Wu and Hu 1938, *R. onychomis* Cuckler 1939, *R. ondatrae* Chandler 1941, and *R. nana* Caballero 1943. *Rictularia nycticebi* may be separated from the present form by its dorsally inclined oral opening. Information as to the comb and spine number of the female is not available relative to *R. vulpis* and *R. muris*, each of which was described on the basis of male specimens. The knowledge of both these is inadequate and their further discussion here is not warranted. Although Travassos (1928) described the females of *R. elegans*, he neglected to mention the numbers of combs and spines. *Rictularia dipodomis* differs from *R. elegans* in that the last pair of spines on the female of the latter are only 0.42 mm from the tip of the tail, whereas in the new species they are located 2.15 mm from the posterior end. *Rictularia oligopectinea* is described as having only 41 to 51 pairs of combs and spines and *R. onychomis* has a maximum of 64 pairs. Only 32 pairs of processes are present anterior to the vulva of *R. ondatrae*, and the probable synonymity of *R. nana* with *R. macdonaldi* has already been pointed out (Tiner 1948). This last mentioned species has only 30–32 pairs of cuticular projections anterior to the vulva, according to the table of Dollfus and Desportes.

As suggested in the above discussion, the writer considers the angle of inclination of the oral opening a valuable criterion for separating certain species of *Rictularia*. It shows very little variation within living or fixed specimens belonging to a single species. Within the genus, the position of the oral opening ranges from almost directly dorsal, as in *R. citelli* McLeod 1933, to almost exactly anterior, as in *R. dipodomis*.

Thanks are expressed to Dr. Price for the loan of the otherwise inaccessible paper by Schulz (1927), and for helpful suggestions. At the outset of this study, Dr. B. B. Morgan, University of Wisconsin, contributed bibliographic assistance by making available an unpublished host list of the genus *Rictularia*. The writer is grateful to Dr. L. J. Thomas, University of Illinois, for suggestions that facilitated the preparation of the plate.

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Explanation of Plate

Morphology of *Rictularia dipodomis* n. sp.

All drawings were made with the aid of a camera lucida. Scales for Figs. 1, 2, and 4 represent 100 μ; scale in Fig. 3, 20 μ; in Fig. 5, 50 μ.

Fig. 1. Diagrammatic side view, anterior extremity of female with one row of combs torn loose.

Fig. 2. Ventral view, female, showing 23rd–27th comb pairs. Right comb of 28th pair missing because of damaged condition of specimen.

Fig. 3. *En face* view, anterior end of female.

Fig. 4. Combs and spines in vulvar region of female. Embryo omitted from third egg.

Fig. 5. Tail of female showing anal opening, and egg filled uterus. Eggs shown in outline form only—embryos omitted.

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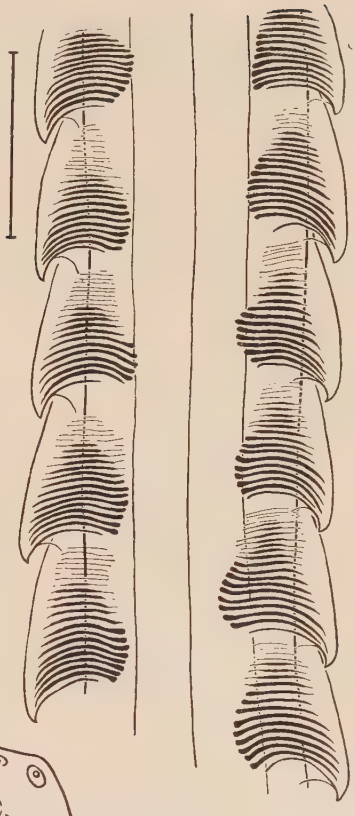

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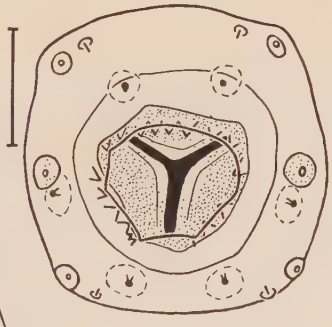

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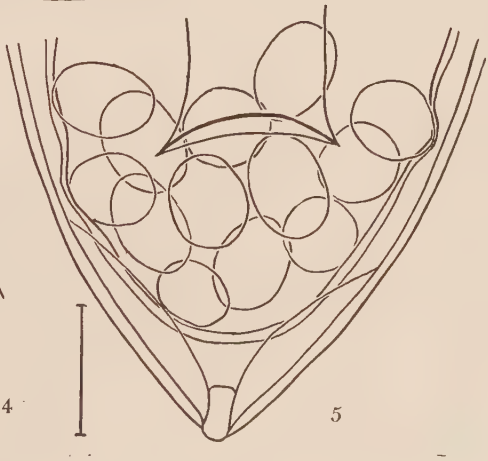

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MYOBIID MITES (ACARINA: MYOBIINAE) FROM SHREWS (MAMMALIA: SORICIDAE) OF EASTERN NORTH AMERICA

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In the course of some small mammal studies in central New York and southern Ontario a collection of ectoparasites was made. This paper discusses one group of these parasites, the mites of the subfamily Myobiinae (Cheyletidae) which were collected from shrews (*Sorex cinereus* Kerr, *S. fumeus* Miller, and *Blarina brevicauda* (Say)). Four species of myobiid mites were found, two of them being here described as new.

Ewing (1938) separated the myobiid mites as a subfamily of the Cheyletidae; and from the genus *Myobia* Heyden he removed several species, establishing for them three new genera. As generic characters the number of segments of the first pair of legs and the number of tarsal claws on the second pair of legs were used. The shape of the penis may also prove to be of assistance in defining genera or species groups; this has been previously used as a specific character. The classification of the dorsal setae as submedian and lateral is followed here as proposed by Ewing (*op. cit.*: 181–183). In addition the setae about the anus are here employed taxonomically, and are termed circumanal setae.

Two species of *Protomyobia* Ewing, 1938 were found, and these may be separated by the following key:

A. Female: submedians III–V long, inflated, III and IV overlapping the bases of IV and V respectively (III extending to the base of V); circumanals I subequal to submedians III–V. *Male:* submedians II meso-caudad from laterals II; submedians IV less than twice the length of submedians III; distances between the bases of laterals I and II subequal to the distances between the bases of laterals III and IV ..... *claparèdei* (Poppe, 1896).

AA. *Female:* submedians III–V very short, not inflated, not overlapping; circumanals I four times as long as submedians III–V. *Male:* submedians II mesad and slightly cephalad from laterals II; submedians IV twice as long as submedians III; distances between the bases of laterals I and II twice the distances between the bases of laterals II and III. *brevisetosa,* n. sp.

Protomyobia claparèdei (Poppe, 1896)

Leg I with five segments as illustrated (Fig. 1, A and B); segments II and III with grooved tubercles venerally; segment V with two curved, terminal claws. Sometimes these claws lie closely appressed to the apex of the fifth segment; this may account for the statement in Ewing's paper (*op. cit.*: 183) that segment V bears a single claw. Leg II with two tarsal claws, the caudal smaller and notched. Penis stout and curved, without loops.

Female (Fig. 1, A): Submedians I small, on a line connecting the bases of laterals I, closer to their adjacent laterals than to each other; submedians II slightly shorter than submedians I, meso-caudad from laterals II, closer to their adjacent laterals than to each other; submedians III–V subequal, III and IV overlapping the bases of IV and V respectively (III extending to the base of V), each about four times as long as submedians I, and more mesal than I or II; submedians III meso-cephalad from laterals III; submedians VI subequal to submedians I, between the bases of legs IV. Circumanal setae of four pairs: circumanals I twice as long as submedians VI, latero-caudad from them; circumanals II–IV in two caudally diverging rows, slightly longer than submedians I. Laterals I long, simple, extending to the bases of laterals III; laterals II similar to and directly caudad from laterals I, on a line connecting the caudal borders of legs II; laterals III slightly longer than laterals I and II, between the bases of legs III.

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Male (Fig. 1, B): Submedians I small, slightly cephalad from a line connecting laterals I, the distance between them about one-third the distance between one submedian and its adjacent lateral; submedians II slightly shorter than submedians I, meso-caudad from laterals II; submedians III longer than submedians I and II; submedians IV longer than submedians III, meso-

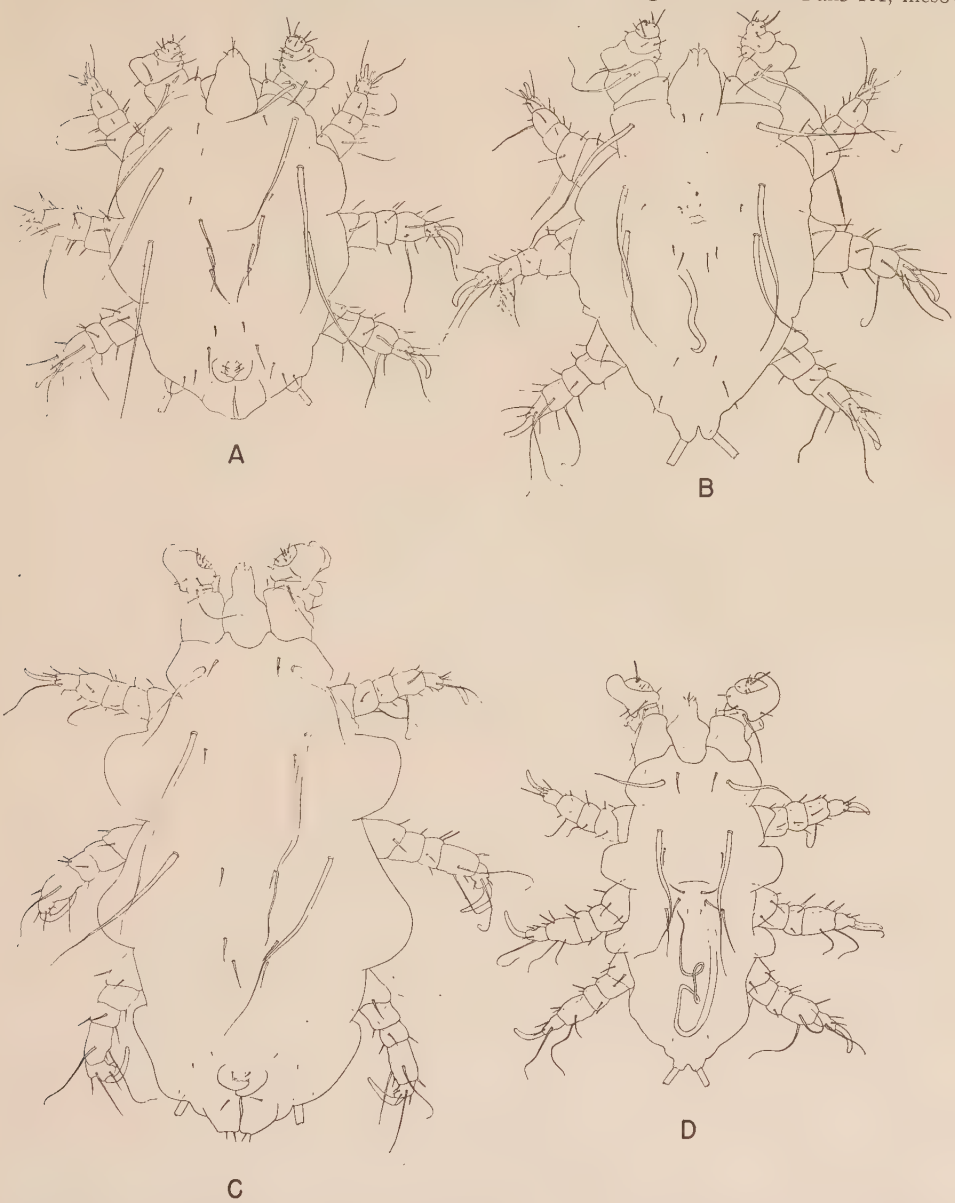

FIG. 1. A, *Protomyobia claparèdei* (Poppe, 1896), dorsal view of female; B, *Protomyobia claparèdei* (Poppe, 1896), dorsal view of male; C, *Myobia simplex* Ewing, 1938, dorsal view of female; D, *Myobia simplex* Ewing, 1938, dorsal view of male.

caudad from them. Circumanal setae of three pairs, in two caudally diverging rows: circumanals I subequal to submedians I and shorter than circumanals II. Laterals I long, simple, extending beyond the bases of laterals III; laterals II similar to and directly caudad from laterals I; laterals III similar to laterals I and II, directly caudad from them. Genital orifice on a line connecting the cephalic borders of legs III, preceded by three pairs of minute setae.

Specimens collected: From *Blarina brevicauda* (Say); Welland County, Ontario, Canada: 21 August 1946, one female; 16 August 1947; three males, two females; 24 August 1947, five females; 27 August 1947, two males, seven females; Tompkins County, New York: 8 November 1946, one male, one female (A. H. Benton); 19 January 1947, one female; 11 February 1947, three females; 15 March 1947, six females; 25 May 1947, one female; 7 June 1947, one female; 24 September 1947, one female; 25 October 1947, one female; 15 November 1947, one male, one female; 16 November 1947, one male; 22 November 1947, one male, two females; 23 November 1947, five females; 6 December 1947, one male. From *Sorex cinereus* Kerr; Tompkins County, New York: 20 August 1947, one female. Above specimens collected by the writer unless noted.

North American specimens of *claparèdei* differ only slightly from the original description by Poppe (1896: 342–344) and from four European specimens loaned by Dr. C. S. Radford: submedians II of the male are meso-caudad from laterals II whereas in the original description of specimens from Europe submedians II lie on a line connecting laterals II. Perhaps additional North American specimens and more extensive host and locality data will show that they ought to be separated as a subspecies. One specimen in the U. S. National Museum from *Sorex fumeus* is somewhat atypical in that submedians III–V are rather short; however they are not at all like submedians III–V of the following species, and in other respects the specimen resembles the above description of *claparèdei.* The occurrence of *Protomyobia claparèdei* on *Sorex cinereus* needs no special explanation inasmuch as the European host is a shrew of the same genus, *S. araneus.* There is a series of seven females collected from *S. fontinalis,* 12 April 1933, Chestertown, Maryland (H. S. Peters) in the U. S. National Museum. This is agreement with the above host distribution as Poole (1937: 96) showed that *fontinalis* is a subspecies of *cinereus.* The presence of *claparèdei* as a common parasite of *Blarina brevicauda* cannot yet be clearly explained; very likely *claparèdei* became a parasite of *Blarina brevicauda* after the evolution of the latter from the primitive soricid stock. This suggestion has indirect support in the presence of the following species on *Sorex fumeus.*

Protomyobia brevisetosa, n. sp.

Leg I with five segments as illustrated (Fig. 2, A and B); legs I and II similar to those of *claparèdei* (Poppe, 1896).

Female (Fig. 2, A): Submedians I small, on a line connecting laterals I, the distance between the adjacent setae being equal; submedians II slightly shorter than submedians I, meso-caudad from laterals II; submedians III–VI increasing in length caudally, subequal to submedians I, more mesal than submedians I or II; submedians III on a line connecting laterals III. Circumanal setae of four pairs: circumanals I four times as long as submedians VI, latero-caudad from them; circumanals II–IV subequal to submedians I, in two caudally diverging rows. Laterals I long, simple, extending to bases of laterals III; laterals II slightly longer than laterals I, directly caudad from them; laterals III subequal to laterals II, directly caudad from them.

Male (Fig. 2, B): Submedians I small, on a line connecting laterals I; distance between submedians I one-third the distance between one submedian and its adjacent lateral; submedians II slightly longer than submedians I, mesad and slightly cephalad of laterals II; submedians III three times as long as submedians I, slightly caudad from a line connecting laterals III; distance between submedians III about equal to distance between one submedian and its adjacent lateral; submedians IV twice as long as submedians III, meso-caudad from them. Circumanal setae of three pairs, in two caudally diverging rows, subequal to submedians I, decreasing in length caudally. Laterals I long, simple, extending beyond bases of laterals III; laterals II similar to and directly caudad from laterals I; laterals III slightly longer than laterals I and II. Genital orifice on a line connecting the cephalic borders of legs II, and preceded by three pairs of minute setae.

Type host: Sorex fumeus Miller (smoky shrew).

Type locality: four miles east of Ithaca, Tompkins County, New York.

Types: Holotype female and allotype male; 20 July 1947; E. W. Jameson, Jr.; deposited in the U. S. National Museum.

Paratypes: One male and one female with same data as types; five males and six females; 18 September 1947, with other data same as types.

Additional specimens: From *Sorex fumeus* Miller; Tompkins County, New York: 4 February 1947, two males, four females; 15 March 1947, ten females; 25 May 1947, four females; 7 June 1947, one female; Greene County, New York: 12 April 1947, two males, six females (W. Robert

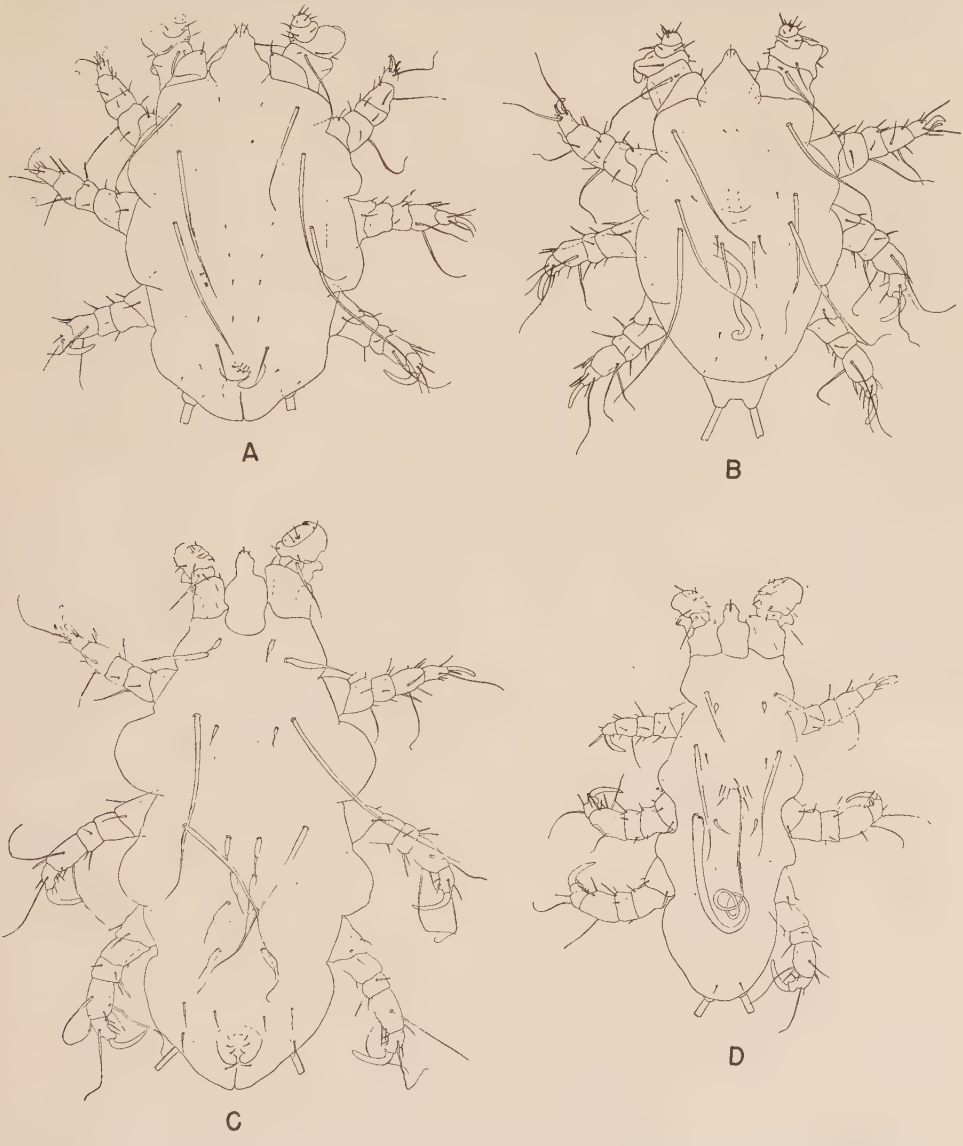

FIG. 2. A, *Protomyobia brevisetosa,* n. sp., dorsal view of female; B, *Protomyobia brevisetosa,* n. sp., dorsal view of male; C, *Amorphacarus hengererorum,* n. sp., dorsal view of female; D, *Amorphacarus hengererorum,* n. sp., dorsal view of male.

Eadie and E. W. Jameson, Jr.); Venango County, Pennsylvania: 24 April 1947, one female (N. D. Richmond and J. J. Christian); 4 May 1947, four males, one female (N. D. Richmond, J. J. Christian). Above specimens collected by the writer unless noted.

A third myobiid mite found on shrews in this study is *Myobia simplex* Ewing, 1938. At the time of its original description, this species was known only from

females. The females collected by the writer agree with the original description except with regard to laterals II which are described as extending to the tips of submedians V. This is a small difference which could be due to individual or geographic variation, or to breakage near the tips of laterals II in the specimens examined by me. The type material consists of three females on one slide; these specimens lack several dorsal setae. Specimens collected by the writer agree with a specimen in the U. S. National Museum identified by Dr. Ewing as *simplex*.

Myobia simplex Ewing, 1938

Leg I with four segments as illustrated (Fig. 1, C and D); in the male there appears to be a vestigial fifth segment. Segments II and III with grooved clasping tubercles ventrally. Leg II with one tarsal claw. Legs I sometimes unequal.

Female (Fig. 1, C): Submedians I small, simple, meso-cephalad from laterals I, each close to its adjacent lateral; submedians II similar to submedians I but shorter, meso-caudad from laterals II; submedians III equal to submedians I, meso-caudad from laterals III, the distances between adjacent setae being equal; submedians III–V increasing in length caudally; submedians V more mesal than submedians III and IV. Circumanal setae of six pairs: two caudally diverging rows of three setae each, all much smaller than submedians I; a pair of setae slightly smaller than submedians I just caudad from the vulva; two pairs of setae at caudal border of anal slit. Laterals I slightly inflated proximally, extending for one-third their lengths beyond bases of laterals II; laterals II long and slender, extending for almost one-half their lengths beyond bases of laterals III, slightly caudad from a line conecting the caudal borders of legs II; laterals III long and slender, extending beyond bases of legs III.

Male (Fig. 1, D): Submedians small, simple, meso-cephalad from a line connecting laterals I; submedians II slightly smaller than submedians I, meso-caudad from laterals II. Genital orifice between bases of legs III; three pairs of setae about genital orifice, two pairs subequal to submedians I, and a much smaller pair. One pair of circumanal setae. Laterals I extending beyond bases of laterals II; laterals II twice as long as laterals I; laterals III about half as long as laterals I.

Specimens collected: From *Blarina brevicauda* (Say); Welland County, Ontario, Canada: 24 August 1946, one female; 1 September 1946, two females; 3 August 1947, one female; 14 August 1947, one male, one female; 16 August 1947, one male; 27 August 1947, one male, one female; Tompkins County, New York: 4 February 1947, one male, one female; 24 September 1947; one female; 22 November 1947, five males, nine females (W. G. Sheldon, E. W. Jameson, Jr.). From *Sorex cinereus* Kerr: Tompkins County, New York: 20 August 1947, one female. Above specimens collected by the writer unless noted.

The fourth species discussed here is that listed as *Amorphacarus elongatus* (Poppe, 1896) by Ewing (*op. cit.:* 191). Specimens collected from *Sorex cinereus* and *S. fumeus* differ from the original description and fine illustrations by Poppe (1896: 344–345). One female collected from a shrew in England (kindly given to the writer by Dr. Charles D. Radford) agrees with the description by Poppe. The above-mentioned North American specimens can be separated from *elongatus* by the following key.

A. *Female:* submedians VI absent; submedians II mesad from laterals II; circumanals I–III in two caudally diverging rows. *Male:* submedians II meso-cephalad from laterals II; genital orifice surrounded by three pairs of small setae. ..... *elongatus* (Poppe, 1896)

AA. *Female:* submedians VI present; submedians II meso-caudad from laterals II; circumanals I–III in two triangles. *Male:* submedians II meso-caudad from laterals II; genital orifice surrounded by four pairs of small setae. ..... *hengererorum*, n. sp.

Amorphacarus hengererorum, n. sp.

As observed by Ewing (*op. cit.:* 192) when he erected this genus, legs I are unequal in size; in the present species it is usually the right leg which is the larger. According to Radford (1935: 252) it is the left leg of *elongatus* which is enlarged. Legs II have two tarsal claws, the caudal claw half the size of the cephalic claw.

Female (Fig. 2, C): Submedians all (except submedians II) scale-like; submedians I

small, meso-cephalad from laterals I, distance between submedians I twice that between one of them and the adjacent lateral; submedians II subequal to submedians I, meso-caudad from laterals II; submedians III and IV equal, slightly longer than submedians I; submedians III mesad and slightly caudad from laterals III; submedians IV directly caudad from submedians III, distance between submedians III and IV slightly less than their length; submedians V and VI equal, slightly longer than submedians III and IV, somewhat more lateral. Circumanal setae of four subequal pairs, simple, not scale-like; first two pairs in a transverse line, the mesal pair slightly more caudal and smaller; circumanals III directly caudad from the outer of the first two pairs; circumanals IV caudad from the vulva. Laterals I somewhat inflated proximally, extending to bases of laterals II; laterals II long and slender, extending beyond the bases of laterals III by one-third their lengths; laterals III equal to laterals II, directly caudad from them.

Male (Fig. 2, D): Submedians I small, scale-like meso-caudad from laterals I; submedians II slightly longer than submedians I, simple, not scale-like, meso-caudad from laterals II; submedians III simple, somewhat longer than submedians II, between the bases of legs III. Genital orifice on a line connecting the cephalic borders of legs III. About genital orifice, four pairs of small, simple setae, some of which may represent the apparently missing submedians IV–VI. Circumanal setae one pair, small, simple, near the caudal border. Laterals I extending to bases of laterals II; laterals II twice as long as laterals I, extending beyond the bases of legs III.

Type host: Sorex fumeus Miller (smoky shrew).

Type locality: four miles east of Ithaca, Tompkins County, New York.

Types: Holotype female and allotype male; 20 July 1947; E. W. Jameson, Jr., deposited in the U. S. National Museum.

Paratypes: Eight males and fourteen females with same data as types.

Additional specimens: From *Sorex fumeus* Miller; Venango County, Pennsylvania: 24 April 1947, one female (N. D. Richmond, J. J. Christian). From *S. cinereus* Kerr; Tompkins County, New York: 7 December 1947, three females (E. W. Jameson, Jr.).

This species bears the name of Barbara T. and David E. Hengerer who have been of great assistance to the writer in field work.

As can be seen from the illustrations (Fig. 2), *Amorphacarus hengererorum* is similar in several respects to *Myobia simplex*. The construction of legs I is essentially the same in both species. *M. simplex* also shows a slight asymmetry in legs I. The penis of *A. hengererorum* resembles that of *M. simplex*. *Amorphacarus* appears to be a slightly aberrant *Myobia,* if *simplex* represents the typical condition of *Myobia*.

Remarks.—The mites discussed in this paper are small, about one-half millimeter long, and so they easily escape the attention of many collectors. It is unusual, however, if at least one or two cannot be found on a shrew. Sometimes, usually in the late winter or spring, a hundred or more of these mites may be present on a single shrew; in such cases the shrew is always an old one. This time of abundance of myobiid mites coincides with a preponderance of old shrews in the population. As pointed out by Hamilton (1940) and Pearson (1945), *Sorex fumeus* and *Blarina brevicauda* are short-lived animals, the majority of them not living more than a year. Apparently the shrews acquire a small initial infestation of myobiid mites which increases continually as the shrews age.

The feeding habits of these mites are not well known. At least one species discussed in this paper (*Myobia simplex*) feeds upon blood: *simplex* can easily be recognized by the red color which shows through the integument. Specimens of adult *simplex* crushed between a glass slide and a cover glass release red blood cells which are the same as those taken from the host; the nymphs do not appear to be blood suckers. Because these mites hang from the tips of the hairs of the dead host and because they sometimes occur in such great numbers, they could easily be picked up by another shrew passing in the same runway, and should therefore be considered as a possible agent in the transmission of an epizootic through a population of shrews.

ACKNOWLEDGMENTS

To Professor Robert Matheson and Dr. V. S. L. Pate I am grateful for advice and for facilities of the Department of Entomology of Cornell University; to Dr. Edward W. Baker for assistance in studying some myobiid mites at the United States National Museum; to Dr. William L. Jellison for carefully reading the manuscript; and to Dr. George Wallace for the loan of myobiid mites from the Carnegie Museum. I wish also to thank E. I. du Pont de Nemours & Company for a supply of polyvinyl alcohol.

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CUTANEOUS MYIASIS IN PANAMA

Carlos Calero M., M.D.[1]

Myiasis of the skin on the Isthmus of Panama was diagnosed for the first time in 1854, according to the bibliography on hand. In the communication of Sullivan (1883) commenting upon the findings of Strain (1854), as well as in Hale's (1903) commenting upon the findings made by G. L. Wickes toward the end of the last century, and later in the communication made by Adams (1904), cases of myiasis of the skin were reported as produced by *Dermatobia hominis* (Linn.).

Since then some other communications have been published, such as Bishop's (1915) and James and Clark's (1915), who reported new cases of myiasis of the skin as produced by *D. hominis* and also the first cases of infection on the Isthmus of Panama as produced by *Chryosomia macellaria* (Fabr.). Dunn's communication in 1918 described a new case of myiasis of the skin as produced by *D. hominis* and reported that the "garrapata" (tick) *Amblyomma cajennense* Koch was possibly the intermediary vector of the eggs. In 1930 Dunn reported that one of the *Limnophora* species was the vector of *D. hominis* eggs in a new case of myiasis of the skin. Finally, in 1934, Dunn reported a case of myiasis of the scalp, of the chest and cerebral myiasis, caused by *D. hominis,* in a girl one and a half years old, and also discussed the frequency of its infection among several animals on the Isthmus of Panama (dogs, cats, rabbits, monkeys, cattle, and sheep).

The foregoing résumé leads to the conclusion that only two types of larvae have been reported as producing myiasis of the skin on the Isthmus of Panama and also that in the majority of the cases the infection was produced by the species *D. hominis,* while the species *C. macellaria* was reported in only a few cases.

The purpose of the present communication is to report two additional cases of myiasis found for the first time on the Isthmus of Panama.

Case I: male, 46 years old, white, referred to us by Dr. J. Feraud P., to study the etiology of a fistulous tract that the patient had presented for a year in the region of the mentum. The patient denied any special subjective symptomatology. The physical examination showed, after removing a thin crust that covered the lesion, that there was a fistulous tract of granular surface with slight peripheral erythema and local edema. The microscopic examination of the crust showed two very small larvae killed by the potassium hydroxide 10% which had been added in the routine test for mycotic organisms. The study showed them to be second stage larvae of *Stephanostoma haemorrhoidalis* (Fall.) = *Sarcophaga haemorrhoidalis.*

Case II: girl, 8 months old, white, with myiasis located on the cranium, was also referred to us by Dr. Feraud P. while I was studying the different types of cutaneous myiasis in Panama (1947) with special reference to incidence and epidemiology. The girl presented a marked agitation, cried constantly and could not rest her head on the afflicted side. Upon local examination an ulceration of 4 × 6 cm was found in the right parietal region which was practically covered by larvae (166 larvae were counted). The border of the ulceration was thick and painless and presented a marked subdermic excavation toward the occipital and temporal regions, six and four cm long, respectively. Upon removing the larvae there were found three points of perforation of the periosteum. The patient did not present either meningeal symptoms or encephalic irritation.

The examination of the larvae and the subsequent study of the flies bred in the laboratory showed that the myiasis was produced by larvae of the species *Cochliomyia hominivorax* (Coq.).

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SUMMARY

The author reports a new case of cutaneous myiasis due to *S. haemorrhoidalis* (Fall.) which is reported for the first time from the Isthmus of Panama.

A first report is made also, but with reservation, of cutaneous myiasis due to *C. hominivorax* (Coq.). Our reservation is due to the fact that for Panama there has been no comparative study since 1933 of the larvae originally reported as *C. macellaria* whereas numerous cases of cutaneous myiasis elsewhere reported before 1933 as due to these larvae proved later to be due to *C. hominivorax*.

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MITE TRANSMISSION OF A HEMORRHAGIC SEPTICEMIA IN SNAKES

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INTRODUCTION

While rearing a culture of snake mites, *Ophionyssus serpentium* (Hirst), on living hosts, it was observed that the hosts died in less than 10 days after being infested. The snakes were kept without water in order to insure a maximum population of mites as the parasites are easily drowned and are repelled by moisture. For this reason, it was thought that the snakes might be dying from lack of water. To check this possibility, mite-free snakes were denied water for a period of 3 weeks, while those infested with mites were given water through a pipette. During this time, none of the mite-free snakes died while the infested snakes continued to die at the same rapid rate, some in as short a time as 3 days. Dissections were made of those that died and cultures were made of the heart blood and material from other organs. In all cases pure cultures of gram-negative rod-shaped bacteria, later identified as *Proteus hydrophilus* (Sanarelli), were obtained from the heart blood and sometimes from the other organs.

For many years the writer, who had had private snake collections since childhood, has thought that there might be some relationship between the constant, but unwelcome, presence of mites in captive snake collections and the relatively high mortality rate of such snakes. The discovery of this highly fatal bacterial disease led to the following research which consists of the identification of the bacterium, a description of the disease, and the mode of its transmission.

MATERIALS AND METHODS

The snakes used for experiment were collected in three Ohio counties: Franklin, Hocking, and Ottawa; or purchased from the Florida Reptile Institute at Silver Springs. These specimens included 3 Blacksnakes, *Coluber constrictor constrictor* Linn.; one Blue Racer, *Coluber constrictor flaviventris* Say; 4 Pilot Black Snakes, *Elaphe obsoleta obsoleta* (Say); 22 Queen Snakes, *Natrix septemvittata* (Say); 9 Common Water Snakes, *Natrix sipedon sipedon* (Linn.); 2 Southern Banded Water Snakes, *Natrix sipedon fasciata* (Linn.); one Florida Water Snake, *Natrix sipedon pictiventris* (Cope); 31 Island Water Snakes, *Natrix sipedon insularum* Conant and

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This paper is from a thesis presented to the Graduate School of The Ohio State University in partial fulfillment of the requirements for the degree of Master of Science. The thesis, containing tables of all original data, is in the Botany and Zoology Library, Columbus, Ohio.

Clay; 2 Brown Water Snakes, *Natrix taxispilota* (Holbrook); 18 Common Garter Snakes, *Thamnophis sirtalis sirtalis* (Linn.); and one Hog-nosed Snake, *Heterodon contortrix* (Linn.). Thus a total of 94 snakes, representing 11 species or subspecies, were used; also 28 white mice and 23 Leopard Frogs, *Rana pipiens* Schreber. These animals were kept in small wooden cages that had screened openings for ventilation and sliding glass tops or fronts to facilitate observations and handling of the specimens within them.

A culture of mites was started with a few hundred specimens which were collected from snakes in the reptile exhibits at the Columbus and the Cleveland Zoos. These parasites were reared on living hosts in a cage which was placed upon a large sheet of white paper. A thick wall of vaseline surrounding the cage and approximately 2 inches out from its sides was applied to the paper. The white paper provided a contrasting background to any dark-colored mites that got out of the cage, thus facilitating their recapture, while the vaseline barrier prevented their escaping and spreading to the remainder of the snake collection. Several 3×3 inch squares of corrugated cardboard, in which the mites could hide, moult, and lay eggs, were placed in the cage.

All snakes that died in the mite-rearing cage were checked for bacterial infection by making smears on microscopic slides and by streaking blood agar slants with the heart blood and material from other organs. If the cultures grew out, they were inoculated into various types of differential media for identification of the organisms. All cultures to be preserved for further experiments were refrigerated at 4° C at which temperature they remained viable and with little loss in virulence for several months. Inoculations of these bacteria were made into healthy snakes, frogs, and mice, using a 1.0-cc tuberculin hypodermic syringe with a 25-gauge needle. Inoculations into snakes were always made into the mouth as this is the only area of the body in which blood vessels are externally visible; however, these blood vessels are small and it was not always possible to place the inoculum directly into the vessel. The injections into frogs and mice were made either intraperitoneally or intramuscularly. In preparing the inoculum, an agar slant culture, 24 to 72 hours old, was used. Ten cubic centimeters of sterile physiological saline were added to this culture and the bacteria were removed from the slant and suspended in the saline with a sterile inoculating loop. The suspension was then transferred to small rubber-capped serum bottles and was ready for use. As these inoculated animals died, they were dissected and grossly examined for pathological effects on the various organs and cultures were made to see if the same bacteria could be recovered.

In working out the percentage of infection with *Proteus hydrophilus* in the mite population, semi-micro qualitative analysis test-tubes were used. Into each of these tubes was placed 0.2 cc of nutrient broth culture medium and a short glass rod with the lower end rounded and the upper end wrapped in non-absorbent cotton. These were autoclaved at 15 pounds pressure for 30 minutes. After cooling the tubes, a single mite, adult or nymph, was placed in each tube and crushed with the glass rod. After 24 hours, the mixture of mite and broth from each tube was poured onto a separate blood agar plate and streaked out with a sterile inoculating loop. After another 24 hours, each plate was examined for hemolytic colonies and a slide of each of these was made and stained, using Gram's method. If the hemolytic colony consisted of gram-negative, rod-shaped bacteria, these were inoculated into differential media to determine if they were *P. hydrophilus*.

DESCRIPTION OF THE DISEASE—A HEMORRHAGIC SEPTICEMIA

The species of bacteria involved in this work was first isolated from frog blood by Sanarelli (1891), who found it to be pathogenic for frogs, toads, salamanders lizards, sunfish, fresh water eels, guinea pigs, rabbits, dogs, cats, chickens, and pigeons. He suggested water as the natural habitat because he found it occurring in 2 of 26 samples of water that he examined. *Proteus hydrophilus* (Sanarelli)[2] has previously been reported as occurring naturally in three species of animals. These are the frog (Sanarelli, 1891), and the brook trout and pike (Reed and Toner, 1942). Kulp and Borden (1942) made an extensive study of the morphological, cultural, biochemical, pathogenic, and immunological properties of the organism as the etiological agent of "Red Leg Disease" in frogs, while Reed and Toner (1942) found *P. hydrophilus* to be the causative agent of "Ulcer Disease" in hatchery-reared brook trout and of "Red Sore" in pike. In these 3 animals, the disease was found to be a hemorrhagic septicemia characterized by general hemorrhagic areas in various parts of the body. Transmission was found to be directly through the water and the organism probably gains entrance into the host through superficial lesions. The suggested means of controlling the disease (Emerson and Norris, 1905, cited from Kulp and Borden, 1942) was to keep the animals at low temperatures, which do not harm the hosts, but do inhibit the growth of the bacteria. The same authors found immunization to be possible but impractical in most cases.

The disease in snakes is essentially the same as it is in other animals in that it is a hemorrhagic septicemia. However, the conspicuous hemorrhages, so characteristic in frogs and fish, are not visible because of the thick, colored integument. One cannot be certain that a snake has had the disease until after observation of typical internal lesions and recovery of the bacteria from the blood. After becoming infected with *P. hydrophilus,* a snake host becomes very sluggish and its normal reflexes, such as rearing back or darting out the tongue when disturbed, slow down considerably or no longer take place. In some cases, the animal seems merely to weaken and die; however, in the majority of cases there are convulsions with much thrashing about in its cage about an hour before death. This violent action continues until a short time before it succumbs. In the last moments before the heart stops beating, there appears to be a gasping for air with the mouth opening and closing.

Gross examinations of snakes dead with the disease showed general hemorrhages in all organs of the body. In most specimens small hemorrhagic areas occurred on the lining of the mouth; there was intense congestion of the blood vessels in the mouth and on the surface of the digestive tract; the kidneys and fat bodies near the kidneys were bloody; and the body cavity was filled with a bloody exudate. The liver always appeared mottled and brownish in color; the lung was very red and usually full of bloody fluid, which also filled the trachea and throat; and the ventricle of the heart was always pale and flaccid, while the auricles were swelled with dark, thick blood.

During the course of this research, 36 mite-infested snakes died. Of this number, 31 showed the symptoms and pathology of a hemorrhagic septicemia and in 28 of these *P. hydrophilus* was recovered in pure culture from the heart blood.

[2] Bergey (1939) gives Chester credit for describing this species; earlier editions give Sanarelli. I have not located any justification for this change.

Over a period of time, 40 other healthy snakes were inoculated with saline suspensions of bacteria from mites or mite-infested hosts in order to test the pathogenicity of the organisms. Five strains of the bacteria were used in these inoculations. Strain "a" was recovered from a Queen Snake, "b" from a Pilot Black Snake, "c" and "d" from two Garter Snakes, and "e" from an infected snake mite. Of the 40 snakes inoculated, 36 died with the symptoms and pathology of the disease and yielded *P. hydrophilus* in pure culture from the heart blood while 4 recovered or were unaffected. Death of the 36 affected animals took place in from 5 hours to 21 days with an average time of 3½ days. Of the 5 strains used in the inoculations, strain "a" killed two snakes in 15 hours and 6 days respectively; strain "b" killed 14 snakes in from 5 hours to 21 days with an average time of 4 days while 2 recovered; strain "c" killed one snake in 16 days while 2 were not affected; strain "d" killed 14 snakes in from 9 hours to 13 days with an average time of 3 days; and strain "e" killed 5 snakes in from 24 hours to 4 days, averaging 48 hours. In all inoculation experiments, controls were inoculated with saline from the same stock solution as the saline used for suspension of the bacteria and in most cases the volume of saline injected into the controls was double the amount of bacterial suspension inoculated into the experimental animals. None of the controls were affected.

Five snakes were fed suspensions of the bacteria orally in an attempt to infect them. A hypodermic syringe was filled with 1.0 cc of a saline suspension of the bacteria, the needle was removed, and the syringe inserted about an inch into the esophagus by way of the mouth and then discharged. None of these animals was affected.

After recovery of the bacteria from the heart blood of snakes dead with the disease, various differential media were used to test the biochemical properties of the organisms so as to identify them. All cultures were reared at room temperature and readings were taken at 24, 48, or 72 hours. The media used and results obtained were as follows: Acid and vigorous gas production in 24 hours in sucrose, dextrose, glucose, maltose, mannitol, dextrin, and arabinose broth; no fermentation after 72 hours in lactose, xylose, inulin, raffinose, dulcitol, and sorbitol broth; napiform liquefaction, 25% after 24 hours and 100% after 72 hours, in gelatin; slight indole formation in tryptone broth; H_2S produced in lead acetate agar; no growth in Koser's citrate agar; acetylmethylcarbinol produced in Vogues-Proskauer broth; nitrites produced from nitrate broth; litmus milk was acid after 24 hours, coagulation and peptonization had started after 48 hours, and peptonization was complete in 72 hours; and blood broth was hemolysed in 24 hours. On nutrient agar plates the colonies were about 2.0 mm in diameter. They were transluscent, grayish to creamy white, raised, circular, butryous in consistency, and the edges were entire. On nutrient agar slants growth was spreading, abundant, and creamy white with a smooth, glistening, raised surface. On blood agar plates the colonies were about 2.0 mm or 3.0 mm in diameter, surrounded by a zone of Beta hemolysis 1.0 mm to 2.0 mm in diameter. They were round, convex, smooth, moist, and glistening with the edges entire. Their color was creamy white in young cultures but gradually turned brown. Growth on blood agar slants was smooth, spreading, raised, glistening, white or gray, and with a wide zone of Beta hemolysis after 24 hours. The growth later became brown and methemoglobin was formed, turning the agar dark green. In nutrient broth there was a dense uniform turbidity with a surface

pellicle in very young cultures and a deposit was present which broke up when shaken. The bacteria were found to be pleomorphic. Long narrow cells, coccobacilli, and long filamentous types were found; but plump, short, encapsulated cells with rounded ends predominated. These occurred either as single or double rods and measured 1.0×2.0 microns. The organisms were gram-negative and showed bipolar staining in most instances. The bacterium has a single polar flagellum and is extremely motile.

These tests, according to Bergey's "Manual of Determinative Bacteriology," proved the organism to be *Proteus hydrophilus* (Sanarelli).

Inoculations were made into frogs and mice, both to confirm the identification of the bacteria as *P. hydrophilus* and to observe the pathogenicity of the organisms for these animals. In both frogs and mice, the organisms appeared to be more virulent than in snakes. Two strains of the bacteria were used on the frogs: strain "a" killed 9 frogs in from one to 16 days with an average time of 5 days; and strain "d" killed 10 frogs in from 3 hours to 6 days with an average time of 42 hours. Saline-inoculated controls were not affected.

Infected frogs, studied in detail, showed the symptoms and pathology typical of "Red Leg," which have been described by various authors. All affected frogs yielded *P. hydrophilus* from the heart blood.

Twenty-four mice used in this work were each inoculated intraperitoneally with 0.1 cc of strain "b" *P. hydrophilus* and all died in from 3 to 39 hours with an average time of 10 hours. Soon after inoculation, the mice became sluggish and huddled together in the corners of the cage with their eyes closed, their fur ruffled, and their bodies trembling. They seemed merely to weaken and die without any apparent convulsions although in the case of 2 pregnant females, abortion occurred just before death. Dissection of the mice revealed that the peritoneal and pleural cavities were both full of bloody fluid. Blood vessels along the vertebral column and on the surface of the digestive tract were intensely congested. The stomach and small intestine were bright yellow in all cases; the liver and spleen were much enlarged; and the ventricles of the heart were pale, while the auricles were generally filled with a bloody exudate that also filled the trachea and extended into the throat in several instances. The 4 saline-inoculated controls were unaffected.

THE SNAKE MITE, *Ophionyssus serpentium* (HIRST)

The snake mite was first incompletely described by Metaxa (1823) in Florence, Italy. Duges (1834) discovered the parasite in France and suggested its relationship to a bird mite, *Dermanyssus avium*. Gervais (1844) also found the mite in France and named it *Dermanyssus natricis*.[3] On the basis of the very minute posterior dorsal plate, Megnin (1884) erected the genus now used, *Ophionyssus*. This name was not generally accepted until recent years because Megnin apparently mistook the narrow, elongate genito-ventral plate for the genital aperture. A complete

[3] The description of this species is too meager to be of much value and one cannot determine, from it, whether *D. natricis* is the same species as *O. serpentium* or not. Hirst (1921) obtained a specimen, believed to have been used by Gervais, and decided that the mites are of similar but distinct species, differing only in the distance between the two dorsal plates and in the shape of the anterior one. The writer, however, is not convinced by Hirst's demonstration and believes that these differences might be accounted for by a variation in the degree of distention of the bodies of the specimens examined, due to engorgement.

description of the adult female was given under the name *Ichoronyssus serpentium* by Hirst (1915), based on specimens collected in the London Zoo. Ewing (1922) erected the genus *Serpenticola* for the parasite, but this he later reduced to synonymy, at the same time establishing the validity of *Ophionyssus* Megnin (Ewing 1929). The first published observations on the life history and habits of the snake mite were by Schroeder (1934). *Ophionyssus serpentium* (Hirst) is a member of the family LAELAPTIDAE, subfamily LIPONYSSINAE, and is the only described species in the genus *Ophionyssus* Megnin.

Hirst (1915) gave the length of the mite as 0.9 mm, while Schroeder (1934) stated that the mites he worked on were approximately 1.3 mm in length and 0.8 mm in width and suggested that those described by Hirst may not have been fully developed. Adult females used in this work measured between 1.0 mm and 1.4 mm in length and from 0.6 mm to 0.9 mm in width. The variation in the size of these mites was probably due to the degree of engorgement, however; while Hirst's specimens may have been, as Schroeder suggested, young females that had not yet fed after moulting.

The small size of the snake mite, especially when immature, makes a mite infestation difficult to detect in its early stages. The fecal deposits, however, are comparatively large and numerous, appearing as round, white specks all over the surface of the snake's scales, and by looking for these a mite infestation can be discovered even without observing the parasites.

The mite is an ectoparasite of snakes and is also occasionally found on lizards. It has been reported at various times from freshly caught reptiles but it is rarely found on wild snakes and those on which it has been collected may have been escaped or released specimens from captive collections. The parasite is a common pest in the captive snake collections of zoos throughout the world and it is thought that the infestations start in new collections probably by means of importations of snakes from other infested collections rather than from freshly-captured wild specimens.

The host of *O. serpentium* in nature, if one exists, is not known. However, from what is known of the habits of the mite, which are those of a typical "nest" parasite, it is inconceivable that it could survive as an obligate parasite of snakes in the wild state. According to Schroeder (1934), it drops from the host before moulting or laying eggs, it lays only about 30 eggs, and it never attaches itself firmly to the body of the snake. There are several reasons why the mite probably would not survive on snakes in nature. Most snakes do not congregate in large groups except during hibernation and for a short time before and after hibernation and even individual snakes do not return to the same spot often enough to support a "nest" parasite. Therefore, after dropping from the host and moulting or laying eggs, the mite would have very little chance of finding a new snake host to attack. For this same reason the young would also find a host only infrequently and since the number of eggs per female is relatively small, the species could not survive the death of a high percentage of young. Snakes ordinarily shed their skins very rapidly and this process, which normally occurs at least once each month or two during the warmer months, usually removes all mites and leaves them in the discarded sack of skin, while the host continues on its way. The mite is repelled by water and is easily drowned. Thus, a swimming snake would lose or kill many of its parasites. These habits, however, which would be so detrimental to the mite on wild snakes, are of

no consequence when they are living on captive reptiles. In a zoological exhibit the snakes are confined in a small space and are, therefore, always easily accessible to the mites which have dropped off the host for one reason or another. Thus *O. serpentium* is well suited for parasitizing captive snakes although it probably could not survive on the same host in nature.

The writer is of the opinion that the original host of the mite may have been either a bird or a rodent, although it has never been reported from either of these animals. This conjecture arises from the fact that *Ophionyssus* is closely related to mites of the genera *Dermanyssus* and *Liponyssus,* which do commonly infest birds and rodents, and from the fact that these animals are the main source of food of many snakes both in nature and in captive collections.

Ophionyssus serpentium is a restless, fast-moving arachnid which travels constantly from one feeding spot to another, never fixing itself permanently as the tick does. It is a blood-sucking parasite and could be, therefore, well fitted for the transmission of a bacterial septicemia.

TRANSMISSION OF THE DISEASE BY THE SNAKE MITE

The possibility of transmission of the disease from snake to snake by *Ophionyssus serpentium* was suspected early in the research, when only snakes infested with mites died of an infection of *Proteus hydrophilus.* To test the possibility of such transmission, a group of healthy snakes was placed in a cage with several bacteria-inoculated snakes and a colony of mites. At the same time, a control group was placed in another cage with inoculated snakes, but without mites. In the control group the inoculated animals were dead with the disease after 2 days, while all of the healthy snakes were still alive and healthy after 3 weeks, at which time they were killed and dissected. In no case were there any symptoms or pathology of the disease, nor was *P. hydrophilus* recovered from the heart blood. On the other hand, in the experimental group, the inoculated animals also died with the disease within a few days, but in this case the healthy snakes, too, began to die after 8 days and were all dead after 16 days. Of the 10 snakes in the experimental group, 8 died in from 8 to 16 days with an average time of 11 days. These animals showed the symptoms of the disease and were heavily infested with mites. Upon dissection the typical pathological effects of a hemorrhagic septicemia were revealed and *P. hydrophilus* was recovered in pure culture from the heart blood. One of the experimental animals died 24 hours after the start of the experiment and showed the pathology of a hemorrhagic septicemia but no bacteria were recovered from the blood; and one died in 12 days, showing no symptoms or pathology of the disease and again *P. hydrophilus* was not recovered from the heart blood.

During the course of this work, 36 snakes died after being infested with mites taken from snakes that had died with the disease. Of these, 31 showed the symptoms and pathology of the disease and *P. hydrophilus* was recovered from the heart blood of 28. The 28 snakes, from which the organism was recovered, died in from 2 to 16 days with an average time of 7½ days, while the 5 that showed the symptoms and pathology but did not yield the bacteria died in from 7 to 24 days with an average time of 19 days. Three other snakes died in from 14 to 24 days, showing no sign of the disease whatsoever.

Although no controlled experiments were conducted to check the effect of the

mites on their host when bacteria were not involved, observations were made on the snakes kept in cages with uninfected mites. These cultures were kept in order to maintain a supply of parasites with which to run experiments on disease transmission. In these cages even very heavily infested snakes lived for at least one month, and in many instances for as long as 4 months, with very little food or water. Dissection of these snakes showed a pronounced anemia in all cases, but in no case were bacteria recovered. It can be concluded, therefore, that the mites themselves can remove enough blood from a snake to cause its death if the host is infested for a long enough time, but death comes much quicker when a bacterial disease is also involved. In his paper on "The Snake Mite," Schroeder (1934) stated that 10 snakes, which were artificially infested with *O. serpentium,* died in from 3½ to 16 days and he attributed the deaths to acariasis. The time of death in these snakes corresponds closely to my observations on death from bacterial septicemia, but seems much too rapid to attribute to acariasis alone. It is suggested that there might possibly have been a bacterial disease involved in Schroeder's experiments.

After the proof of mite transmission of *P. hydrophilus* seemed reasonably conclusive, an experiment was conducted to find out the approximate percentage of mites in the population that were carrying the bacteria. The mites were divided into 3 basic groups: those running free in the cage; those on healthy snakes; and those on snakes infected with *P. hydrophilus.* The mites used were selected at random except that approximately half of them were adults and half were nymphs. Of 90 mites running free in the cage, none were infected; of the 60 mites collected from healthy snakes, only one was infected with *P. hydrophilus;* while 64 of 70 mites taken from infected snakes yielded the bacteria.

These data allow no definite statement of the percentage of infection with *P. hydrophilus* in the mite population but it is believed that they do indicate that *O. serpentium* is merely a mechanical carrier of the disease and probably does not transmit the disease for very long after ingesting infected blood. The snakes do not acquire the disease by accidental ingestion of infected mites and the mite fecal deposits harden rapidly and are not powdery, so fecal contamination of the bite wounds is unlikely. Therefore, the means of infection is probably only thru injection of the bacteria by the mite in feeding. Further investigations will be required to find out if the bacteria are pathogenic for the mites. The length of time that the mites remain infective is not definitely known, but indications are that the period is not more than 24 to 48 hours in duration. This hypothesis is based on one experiment in which only 4 snakes were involved. A snake inoculated with *P. hydrophilus* was placed in a cage with a healthy snake and approximately 100 mites. The inoculated snake died in 24 hours and the other snake also died with the disease 2 days later. Twenty-four hours after the death of the second snake a third snake was placed in the cage. This snake did not die but it did become definitely sluggish after about 24 hours and then recovered completely. A fourth snake was also placed in the same cage 48 hours after the death of the second and showed no sign of infection after a week had passed.

CONTROL OF THE DISEASE IN SNAKES

The best way to control the septicemia in snakes would be to control the vector, *Ophionyssus serpentium,* but, up to the present time, none of the suggested measures

have proved satisfactory. Therefore, until effective and practical methods for checking mite infestations are devised, attempts should be made to prohibit the spread of the disease.

Although the following measures have not been tested on a zoological collection, in the light of data obtained in this research, they appear to be the simplest and most logical steps to take when *Proteus hydrophilus* appears in a snake collection. In order to prevent the spread of the infected parasites, the mite-infested cage should be isolated from other cages by surrounding it with a thick wall of vaseline or oil if it cannot be moved. Then, for at least a week, no snakes should be placed in the cage from which the dead snake has been taken in order to insure the passage of the infective period of the mite. Infested snakes frequently immerse themselves when water is available and in this way they may kill many of the parasites. For this reason, water containers in the enclosures should be kept full at all times. Meanwhile, attempts should be made to eradicate the parasites with various acaricides, making certain to exclude those chemicals toxic to reptiles.

CONCLUSIONS

1. *Proteus hydrophilus* (Sanarelli), which has previously been reported as the etiological agent of diseases in certain species of frogs and fish, is also the cause of a severe and highly fatal hemorrhagic septicemia in snakes.

2. The Snake Mite, *Ophionyssus serpentium* (Hirst), which is a common parasite in zoological collections all over the world, is involved in the transmission of this disease from snake to snake.

3. The mite is probably only a mechanical vector and its infective period is probably less than 48 hours.

4. Only negative results were obtained in experiments designed to transmit the disease from snake to snake in the absence of mites. Attempts to infect snakes by feeding with the bacteria orally were also unsuccessful.

5. Control measures should be directed towards eradication of the mites. However, until such methods are devised, steps can be taken to prevent the spread of the disease by isolating cages containing infected parasites for at least the duration of the infective period.

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